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ON FORCE-FREE MAGNETIC FIELDS

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1. *Introduction.*—The possibility is being increasingly considered that cosmic magnetic fields which occur in regions of low density (such as in stellar envelopes, in gaseous nebulae, and in interstellar space) are force-free[1] in the sense that the Lorentz force

$$L = j \times H \tag{1}$$

(where j denotes the current density and H the strength of the field) vanishes. The fact that the magnetic field in the Crab Nebula has an extraordinarily regular pattern[2] has greatly strengthened this view.

The arguments which are generally advanced to support the view that cosmic magnetic fields may, very largely, be force-free are as follows: ionized stellar material is of such high electrical conductivity that large currents may flow; unless special circumstances are operative, these currents will give rise to a Lorentz force of the order of magnitude H^2/l, where l is a suitable characteristic length; the prevailing pressure gradients or gravitational or inertial forces cannot balance a Lorentz force of this magnitude; consequently, the Lorentz force must vanish.

The vanishing of the Lorentz force clearly requires that the current, j, flows everywhere parallel to the magnetic field; and, since $4\pi j = \text{curl } H$, an equivalent expression of the same condition is

$$\text{curl } H = \alpha H, \tag{2}$$

where α is some function of position, arbitrary except for the requirement

$$\text{grad } \alpha \cdot H = 0, \tag{3}$$

which follows from taking the divergence of equation (2).

The arguments advanced to support the view that the magnetic fields (under the circumstances contemplated) are force-free do not give any clue as to what determines the function α. For this reason, the case α = constant, which has mostly been considered in the literature,[1, 2, 3] would appear excessively *ad hoc*. Indeed, if the force-free character of cosmic magnetic fields, in the large, is accepted, questions of two different kinds arise. First, will the magnetic field in an isolated piece of highly ionized matter (in the absence of external forces) tend to a force-free state from an arbitrary initial state? (Closely related to this question is, of course, the

stability of force-free configurations.[4]) Second, if the approach to a force-free state is in accordance with some general principle, then how does the function α become determinate? We shall consider these questions from some general points of view.

2. *The Condition for Maximum Magnetic Energy for a Given Mean-Square Current Density.*—As we have explained, arguments for the force-free character of cosmic magnetic fields are largely derived from the inferred prevalence of currents. In the limit of infinite electrical conductivity, which is usually considered in these connections, the mean-square current density (in the absence of motions) cannot change; we may therefore regard it as given. On the other hand, in a medium of infinite electrical conductivity, one may envisage processes by which an initial magnetic field is amplified. Accordingly, we may ask: *What is the maximum magnetic energy a plasma can have for a given mean-square current density?*

It is to be observed, that, for a given mean-square current density, we can ask only for the maximum magnetic energy, *not* the minimum. For the current density depends on the gradients of the field, and these can be made arbitrarily large without increasing the absolute value of the field.

We ask, then, for the maximum of

$$\int_V |\boldsymbol{H}|^2 \, dV$$

(where the integral is extended over the volume, V, of the plasma under consideration) for a given value of

$$\int_V |\text{curl } \boldsymbol{H}|^2 \, dV.$$

As a problem in the calculus of variations, this requires

$$\int_V \delta\boldsymbol{H}\cdot\boldsymbol{H} \, dV = 0, \tag{4}$$

for all variations $\delta\boldsymbol{H}$ for which

$$\int_V \text{curl } \boldsymbol{H}\cdot\text{curl } \delta\boldsymbol{H} \, dV = 0. \tag{5}$$

By an integration by parts, the integral in equation (5) becomes

$$-\int_S \text{curl } \boldsymbol{H}\cdot(\delta\boldsymbol{H} \times d\boldsymbol{S}) + \int_V \delta\boldsymbol{H}\cdot\text{curl } (\text{curl } \boldsymbol{H}) \, dV, \tag{6}$$

where S denotes the bounding surface and $d\boldsymbol{S}$ is an element of this surface. The surface integral in (6) vanishes when one of two conditions is satisfied: either the variations in the field considered do not alter it on the boundary (in which case $\delta\boldsymbol{H} = 0$ on S), or there are no surface currents (in which case curl $\boldsymbol{H} \times d\boldsymbol{S} = 0$). We shall suppose that one of these conditions obtains and that the surface integral vanishes. We shall then be left with

$$\int_V \delta\boldsymbol{H}\cdot\text{curl } (\text{curl } \boldsymbol{H}) \, dV = 0. \tag{7}$$

By making use of a Lagrangian multiplier, α^2, we can combine equations (4) and (7) to give

$$\int_V \delta\boldsymbol{H}\cdot\{\text{curl } (\text{curl } \boldsymbol{H}) - \alpha^2\boldsymbol{H}\} \, dV = 0, \tag{8}$$

where $\delta\boldsymbol{H}$ may now be regarded as an entirely arbitrary variation. We must, therefore, have

$$\text{curl (curl } \boldsymbol{H}) = \alpha^2 \boldsymbol{H}. \tag{9}$$

The solutions of equation (9) include all force-free fields with constant α; but the class of all solutions of equation (9) is much wider.

In solving equation (9) we may certainly use the vanishing of the normal component of the current, $\boldsymbol{j}\cdot d\boldsymbol{S}$, as a boundary condition: for no internal motions in an isolated plasma can generate a current normal to the boundary. But the boundary condition on $\boldsymbol{j}\cdot d\boldsymbol{S}$ will not suffice to determine the field completely. When magnetic fields belonging to this wider class of solutions of equation (9) are present, they will exert a force on the plasma and generate motions; these in turn will react on the field and alter its character. Only when the Lorentz force vanishes will the magnetic field continue to have the extremal property and be stationary. We have thus shown the following:

In a piece of highly ionized matter (which may be a part of a larger piece) a state of maximum magnetic energy (per unit volume) for a given mean-square current density (exclusive of surface currents) can exist in a stationary state only if the magnetic field is force-free with a constant α.

It is to be specially noted that the foregoing does not prove that the state of maximum magnetic energy will necessarily be attained. All it proves is that, *if* the state is attained and is further stationary, then it will be a force-free state with a constant α. The theorem leaves open the question whether stationary states of nonconstant α with lower magnetic energy can prevail.

3. *The Case of an Isolated Spherical Plasma.*—The manner in which the stationary state envisaged in Section 2 may come to be established can be described a little more explicitly by considering an isolated spherical plasma.

The general solution of equation (9) can be expressed as a linear combination of certain poloidal and toroidal fields defined, for example, by Chandrasekhar and Kendall equations (13) and (14).[3]

The general solution of equation (9) which satisfies the boundary condition $j_r = 0$ can be written in the form

$$\boldsymbol{H} = \sum_{m=0}^{n} A_n^m \left\{ p_n^m \boldsymbol{T}_n^m + q_n^m \boldsymbol{S}_n^m \right\}, \tag{10}[5]$$

where p_n^m and q_n^m are constants and the summation in equation (10) is over m only. Moreover, in equation (10) the radial functions occurring in $\boldsymbol{T}_n^m$ and $\boldsymbol{S}_n^m$ are all defined in terms of the Bessel function $J_{n+1/2}(\alpha_j r)$, where α_j is a zero of $J_{n+1/2}(x)$. Since the energies associated with $\boldsymbol{T}_n^m$ and $\boldsymbol{S}_n^m$ are equal, it is clear that solutions leading to the same total energy will be obtained if

$$(p_n^m)^2 + (q_n^m)^2 = \text{constant}. \tag{11}$$

The field defined by equations (10) and (11) will interact with the medium and will not be stationary. A stationary state will be reached only when p_n^m and q_n^m become equal; the field will then be force-free, and there will be equal amounts of energy in the poloidal and toroidal components.

It should be emphasized that the field described by equations (10) and (11) requires surface currents on the boundary. For varying coefficients p_n^m and q_n^m compatible with condition (11), the volume integral of $|\boldsymbol{H}|^2$ and $|\text{curl } \boldsymbol{H}|^2$ are the

same; but the amounts of surface currents required are different. When allowance for the surface currents is made, the force-free state appears as a state of lower magnetic energy relative to the maximum.

4. *The State of Minimum Dissipation for a Given Magnetic Energy.*—An alternative formulation of the principle of Section 2 is the following:

Consider a plasma in which there is a certain amount of magnetic energy. Let the plasma be characterized by a certain ohmic resistance, λ. We ask: *What is the state of minimum dissipation of magnetic energy by Joule heating?* The problem here is the minimization of

$$\int_V \lambda \left|\text{curl } \boldsymbol{H}\right|^2 dV,$$

for given

$$\int_V \left|\boldsymbol{H}\right|^2 dV.$$

Clearly, this leads again to equation (9) if λ is assumed to be a constant throughout the plasma and if the same assumptions regarding the vanishing of the surface integral in (6) are made. And, as in Sections 2 and 3, we may now argue in terms of the principle of the minimum dissipation of energy, for the occurrence of force-free magnetic fields with a constant α.

It is worth recalling, in this connection, a fact to which Lundquist[6] first drew attention, namely, that force-free magnetic fields with a constant α are the only stable forms of magnetic fields in a uniform plasma which can decay without causing material motions in the plasma. The relevance of this remark for the attainment of a force-free state is the following: For a given amount of magnetic energy, the states of minimum dissipation are governed by equation (9). The solutions of this equation include force-free fields, but they are more general. When the magnetic field belongs to the wider class of solutions, it will induce motions which will result in an *increased* dissipation. Only the force-free solutions of equation (9) characterize states of truly minimum dissipation. Thus the thermodynamic requirement of minimum dissipation[7] leads to the mechanical characterization of the fields as force-free with a constant α.

The foregoing arguments are based on the assumption that dissipation takes place by pure ohmic resistance. But it is known[8] that in "cold" plasmas the conductivity is a tensor and that there is a contribution to the dissipation which is proportional to the volume integral of $\left|\boldsymbol{j} \times \boldsymbol{H}\right|^2$. This contribution to the dissipation is clearly minimized when $\boldsymbol{j}$ is parallel to $\boldsymbol{H}$. If we include this $\left|\boldsymbol{j} \times \boldsymbol{H}\right|^2$ term in the dissipation, the force-free character of the field and the constancy of α both follow directly from a principle of minimum dissipation of energy.

The usefulness of the arguments in this section depends on the relaxation time for the attainment of states of minimum dissipation. Since the term proportional to $\left|\boldsymbol{j} \times \boldsymbol{H}\right|^2$ mentioned in the preceding paragraph is often (in astronomical connections) the most important, it is clear that the principle of minimum dissipation of energy will be most efficient in the establishment of a force-free state with a constant α.

5. *Concluding Remarks.*—The two principles formulated in Sections 2 and 4 are in many ways complementary. Thus, if we consider a plasma with a finite electrical conductivity, the states with minimum dissipation (for a given magnetic

energy) are the force-free states with a constant α; they are therefore the states most likely to survive on thermodynamic grounds. On the other hand, for a plasma in which the decay of the magnetic field is not significant and processes of magnetic amplification can be present, the force-free state is also a likely one, in that it can be reached by a decrease of energy (if we allow for the surface currents) from a state of higher magnetic energy. And it is important to note that, from both points of view, the maintenance of a stationary state requires the appearence of surface currents at the boundaries.

That surface currents are necessary in the case when there is no field external to the plasma can also be inferred from the virial theorem.[9] When no gravitational or pressure effects are present, the theorem requires

$$\frac{d^2}{dt^2}\int_V r^2 \rho dV = \frac{1}{8\pi}\int_V |\boldsymbol{H}|^2 \, dV. \tag{12}$$

The energy in the field, therefore, tends to expand the configuration independently of the geometrical character of the field. If force-free fields without surface currents were possible, then a force would be exerted nowhere, and we should have a contradiction with the requirements of the virial theorem. However, the existence of surface currents and the force exerted on them by the field resolve the apparent paradox.

We can avoid surface currents on a plasma with a force-free magnetic field by fitting suitable external fields. Thus Lüst and Schlüter[1] have shown how an axisymmetric force-free field surrounding a star can be attached in a continuous way to a field which asymptotically becomes uniform at large distances; however, the field at infinity must be parallel to the axis of symmetry of the field in the neighborhood of the star. It may not be possible to meet this latter condition in all cases; for example, a rotating star may have a force-free field surrounding it; and we may expect the field to be axisymmetric about the rotational axis; and, since there is no reason to suppose that the rotational axis is in the direction of the galactic field, we cannot attach the force-free field surrounding the star to the interstellar fields without boundaries on which surface currents are present, or regions in which the force-free condition is not satisfied.

[1] R. Lüst and A. Schlüter, "Kraftfreie Magnetfelder," *Z. Astrophys.*, **34**, 263–282, 1954.

[2] L. Woltjer, "The Crab Nebula," *Bull. Astr. Netherlands*, **14**, 39–80, 1958.

[3] S. Chandrasekhar, "On Force-free Magnetic Fields," these PROCEEDINGS, **42**, 1–5, 1956; S. Chandrasekhar and P. C. Kendall, "On Force-free Magnetic Fields," *Astrophys. J.*, **126**, 457–460, 1957.

[4] S. K. Trehan, "On the Stability of Force-free Magnetic Fields," *Astrophys. J.*, **126**, 429–456, 1957; L. Woltjer, in preparation.

[5] Note that the field given by equation (10) satisfies the identity

$$\text{curl } \boldsymbol{H}\cdot(\boldsymbol{H}\times\boldsymbol{I}_r) = 0.$$

[6] S. R. De Groot, *Thermodynamics of Irreversible Processes* (Amsterdam: North-Holland Publishing Co., 1952).

[7] T. G. Cowling, *Magnetohydrodynamics* (New York: Interscience Publishers, Inc., 1957), see pp. 111–112.

[8] S. Lundquist, "Magneto-hydrostatic Fields," *Arkiv Fysik*, Vol. **2**, No. 35.

[9] E. Fermi and S. Chandrasekhar, "Problems of Gravitational Stability in the Presence of a Magnetic Field." *Astrophys. J.*, **118**, 116–141, 1953.

*MONTHLY CYCLES IN AN ORGANISM IN CONSTANT CONDITIONS DURING 1956 AND 1957**

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The living organism is a very delicately poised, sensitive system. Cycles of several cosmic frequencies—solar-day, lunar-tidal, fortnightly, monthly, and annual—have long been known to occur in numerous plants and animals under natural conditions. And during the last half-century, solar-day and lunar-tidal, lunar monthly, and even annual cycles have been reported to persist in constant conditions in the laboratory. In recent years it has become quite firmly established that these cycles in constant conditions usually have their frequencies essentially temperature-independent and may even have them apparently wholly temperature-independent (daily cycle of fiddler crab, 6°–26° C.);[1] lunar-tidal cycle of fiddler crabs, 13°–30° C.;[2] daily cycle of oat seedling growth, 16°–28° C.;[3] annual cycle of germination in dried seeds, −22 to +45° C.[4]

The evidence is now overwhelming to indicate that organisms, even in "constant" conditions, are deriving cycles of the natural geophysical frequencies from the external environment. These, it is postulated, they use in the maintenance of highly labile, non-phase-locked, more or less frequency-precise cycles of their numerous biological activities.

In the course of an investigation of the character of fluctuation in O_2-consumption in constant light and temperature in three species of plants—the potato, carrot, and seaweed, *Fucus*—during the spring and summer of 1954, Brown, Freeland, and Ralph[5] found in each instance that the average rate of O_2-consumption was higher during the fortnight centered on full moon than during that centered on new moon. For the potato the difference was 14 per cent; the carrot, 11 per cent; and *Fucus*, 6 per cent. In addition, it was noted that in the form of the average daily cycles, the ratio of morning to afternoon rates was higher over the period of full moon than over that of new moon. A quite similar monthly cycle of changing ratio of rates of O_2-consumption for the two halves of the day was reported by Brown, Webb, Bennett, and Sandeen[6] for two species of fiddler crabs and a salamander.

During the above-mentioned studies, it was demonstrated that correlations highly significantly different from zero occurred between the hourly metabolic rate and the concurrent hourly barometric-pressure changes. The pressure changes during the studies had not only clear mean daily cycles but appeared also to exhibit a synodic monthly cycle in the form of the daily cycles.

Experiments and Results.—In the results to be reported now, potatoes in constant conditions, including pressure, in automatic, continuous-recording respirometers were studied for 13 of the 14 months, February 1, 1956, through March 31, 1957. The type of respirometer used has been described by Brown.[7, 8] Five independent respirometer ensembles, each with four cores of potatoes bearing eyes were run continuously. The respirometers remained sealed and undisturbed for periods ranging usually from 2 to 6 days (av., 4.1). They were opened and the

organisms exposed to room conditions for only 15–25 minutes, irregularly at these long intervals for purposes of maintenance of the respirometers. The same individual growing plants were retained as long as possible, in some cases even up to several months.

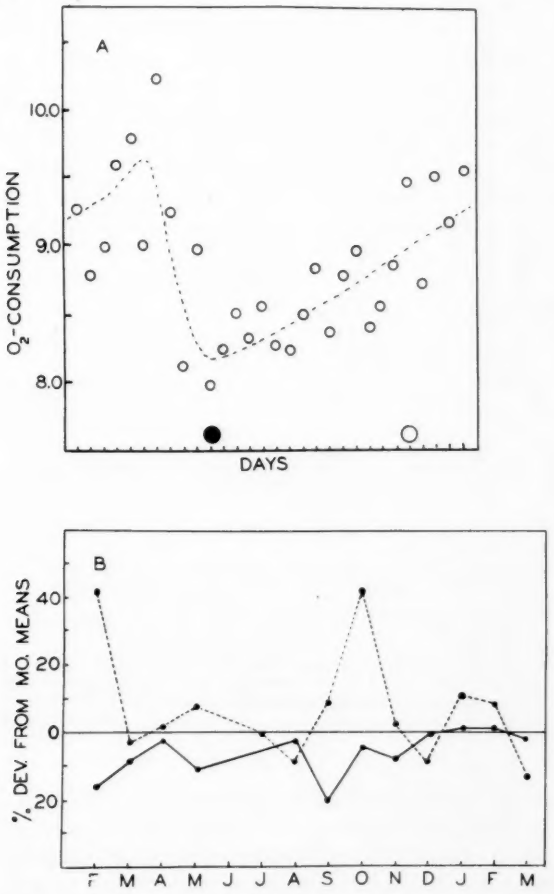

FIG. 1.—*A*, Average rate of O_2-consumption of potatoes as a function of phase of moon. Twelve months of data were synchronized relative to the day of new moon (*solid circle on abscissa*). *B*, The mean per cent deviations from monthly means of the 10-day periods, new moon through new moon +9 (*solid line*) and new moon +17 through new moon +26 (*broken line*), for each period studied, the latter indicated approximately by months of year (*abscissa*).

Twelve uninterrupted synodic monthly periods of hourly data were obtained and analyzed. There were evident from these data mean lunar monthly cycles. These were seen for four more or less independent parameters of O_2-consumption. The first of these was simple *average daily metabolic rate*. Figure 1, *A*, depicts the aver-

age for all data for the 12 lunar months. The minimum rate was seen at the time of new moon; the maximum rate was 4–5 days prior to new moon. Expressed as percentage deviations from synodic monthly means, the difference in rate between the 10-day period, new moon through new moon +9, and the 10-day one, new

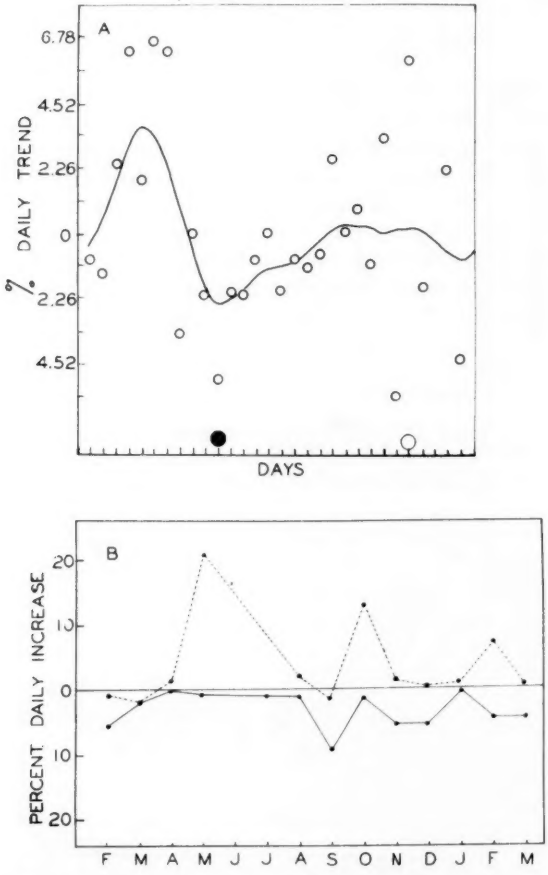

FIG. 2.—*A*, Average daily increase in O_2-consumption expressed as per cent daily trend as a function of phase of moon. Thirty-day periods of data are synchronized by the day of new moon (*solid circle on abscissa*). *B*, The mean daily increases for 5-day periods, day before through 3 days after new moon (*solid line*) and new moon −8 through new moon −4, for each period studied, the latter represented by the approximate calendar months (*abscissa*).

moon +17 through new moon +26, was 11.4 ± 2.6 per cent. The variability of the cycle from month to month may be seen in Figure 1, *B*. The dashed curve indicates the third-quarter period values, the solid line the new-moon period ones.

A second type of lunar monthly cycle was seen in *acceleration in rate* of O_2-con-

sumption following the sealing of the respirometers after each brief (15–25 minutes) exposure of the plants to room illumination of about 500 lux. This was measured as the daily increase in O_2-consumption expressed as a percentage of the daily mean. In Figure 2, *A*, one finds this acceleration shown as a function of day of lunar month. The difference between the maximum acceleration 4–8 days before new moon and minimum acceleration from 1 day before new moon to 3 days afterward was 7.0 ± 2.2 per cent. Expressed in other terms, the mean daily increase for the 5-day period over new moon was found to be 5.3 per cent, and for the 5-day period over third quarter, 12.3 per cent. This actually involved, therefore, a 2.3-fold difference. This monthly cycle was superimposed on an apparent annual fluctuation, exhibiting a 5.7-fold difference.[9] The variation from month to month in this cycle may be seen in Figure 2, *B*. The broken line indicates the third-quarter period values; the solid line, the new-moon period ones.

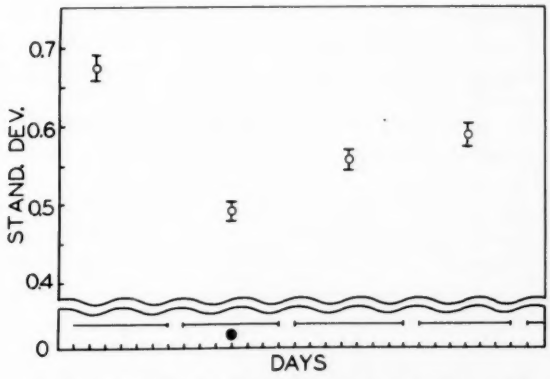

FIG. 3.—The standard deviations and their standard errors for all measurements of O_2-consumption taken at nine times of day, for each of four quarterly periods of the month (*horizontal bars along abscissa*). Day of new moon shown by solid circle on abscissa.

A third way in which synodic monthly cycles were apparent was in the *standard deviation of O_2-consumption measurements.* To illustrate this, the standard deviation for O_2-consumption measurement was obtained at 9 hours of the day (1, 3, 6, 9, 12 A.M.; 3, 6, 9, 12 P.M.) for four quarterly periods of the lunar month, centered on each of the quarters of the moon. These, with their standard errors, are depicted in Figure 3. It is clearly evident that the standard deviation of data collected during approximately 1 week over the third quarter of the moon is very highly significantly greater than that obtained during the week over new moon. Intergrading values are seen during the other two quarters of the moon.

A fourth way in which synodic monthly cycles were demonstrable was in the *form of the daily cycle* in O_2-consumption. In Figure 4, *A*, is seen the mean form of the daily cycle in the potato for the period from February, 1956, through March, 1957, expressed as deviations from a 1 A.M. to 12 midnight linear daily trend. In Figure 4, *B*, the results for 7 (or 8) days centered approximately on each of the four

quarters of the moon are shown. As suggested by the observations published in 1955, the A.M. values are relatively low compared with the P.M. ones over the period of new moon (*lower broken curve*) and relatively higher at third quarter (*higher broken curve*). The two intervening weeks exhibit intergrading values. The lower solid curve was obtained for first quarter, and the higher solid one for full moon.

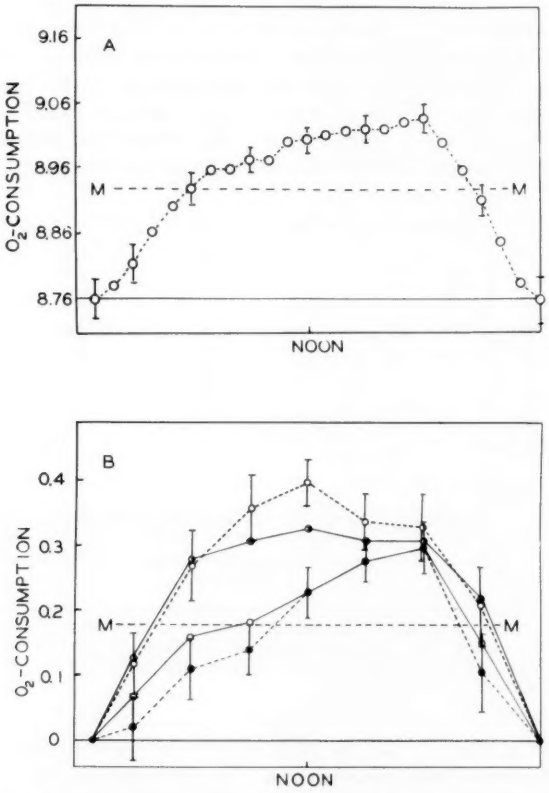

FIG. 4.—*A*, The mean daily deviation of O_2-consumption in the potato, from linear daily trend with standard errors (plotted on mean absolute-rate ordinate) for the total, essentially 13-month, period of study. *B*, Mean daily cycles as in *A*, but now calculated as a function of 7-day (or 8) periods centered approximately on each of the four quarters of the moon. New moon, *lower broken line;* first quarter, *lower solid line;* full moon, *upper solid line;* third quarter, *upper broken line.*

The standard errors are shown only for the new-moon and third-quarter periods. The errors are approximately of the same size for the other two periods. It is quite evident that the fortnight including full moon and last quarter yields a much higher-amplitude daily cycle than does the alternating fortnight and that the difference between them is highly significant. This difference would appear very prob-

ably due to the changing ratio, positive/negative, of the signs of the early morning response[8] to the still unidentified, but clearly demonstrated, external effective variable.

Discussion.—The foregoing results demonstrate clearly that during the period of this investigation the potatoes possessed a statistically significant average synodic monthly cycle while in conditions which were constant with respect to all external factors usually considered to influence them. Since for the potato tuber such a monthly cyclicity has no currently conceivable adaptive significance, it seems probable that there are cycles of this frequency in some external physical factor, to which a living thing such as the potato may respond, even while maintained in so-called "constant conditions." In support of this view are the recent reports of lunar monthly cycles of O_2-consumption and spontaneous activity of earthworms and of spontaneous activity of the salamander, *Plethodon cinereus*, in constant illumination and temperature.[10] In these instances, too, an adaptive significance of cycles of this frequency is unknown. On the other hand, in the shrimp, *Anchistioides*, in which a synodic monthly cycle of molting occurred even while the animals were maintained inside the laboratory in aquaria,[11] an adaptive character of the cyclicity is evident in its relationship to reproductive synchrony within the species and is associated with the normal lunar monthly swarming rhythm in this shrimp.

There was clear proof presented through discovery of correlations significantly different from zero between the potato metabolism and concurrent *aperiodic* fluctuations in forms or amplitudes of the daily cycles of both barometric pressure and background radiation[8, 12, 13] that these organisms in the conditions of constant illumination, temperature, humidity, oxygen tension, pressure, etc., were still responding to some pressure- and radiation-correlated external physical variable during those large fractions of the 13-month period of this investigation for which possible correlations were analyzed.

Just as synodic monthly cycles may, of course, be simple derivatives, by periodic reinforcement, of precise persistent organismic solar-day and lunar-day cycles, so does this study provide further support for the reported simultaneous occurrence of persistent, metabolic, solar- and lunar-day periodisms in organisms as diverse as crabs, salamanders, potatoes, seaweed, and carrots.

Inasmuch as one of the ways in which the synodic monthly cycles become evident is through a modulation in form of the daily cycles, it would seem reasonable to postulate on this basis that there is some common denominator in the fundamental mechanisms of maintenance of these two cycles of widely different frequency, which persist in the so-called constant conditions.

The character of the lunar modulations of the daily cycles are quite consistent with the view that the influences of the sun and moon are qualitatively and temporally similar, but with the sun exerting the greater influence. If in the mean solar-day cycle (Fig. 4, *A*), we interpret the form as a consequence of solar influence on the daily cycle to be one of maximal O_2-consumption about 6 hours later in the day than solar zenith, then the forms of the lunar-modified daily cycles (Fig. 4, *B*) can be interpreted on the basis of a comparable temporal relationship of time of lunar zenith to maximal lunar influence. At new moon, with zenith at solar noon, the maximal lunar influence would be expected near 6 P.M. At first quarter, with zenith near 6 P.M., the maximum influence would be near midnight. At full moon,

the maximal lunar influence would be expected to center over 6 A.M. and, at third quarter, to center over the noon hour. These expectations are essentially fulfilled. This qualitative similarity relative to bodies intrinsically as different in most respects as our sun and moon might suggest that gravitational forces in some manner enter into the chain of forces coupling them with biological activities, possibly through certain geophysical fluctuations associated with the well-known solar and lunar tides of the atmosphere.

In summary, it has been shown that during the 14-month period when this study was being made, there were statistically significant synodic monthly cycles of O_2-consumption, with a skewed form. This was demonstrated for four more or less independent parameters of metabolic change which were found to exhibit among themselves only low correlations in day-to-day changes. In each instance, in the average skewed monthly cycle for this period, the minimum occurred at the time of new moon, and maximum at the time of third quarter.

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*DEVELOPMENT OF COLLAGEN IN THE FROG EMBRYO**

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INTRODUCTION

Ideally, any macromolecular substance which is selected as a model for a study of embryonic chemodifferentiation should satisfy the following criteria: The substance should be identifiable by at least one highly specific and sensitive analytical method, preferably chemical. It should be well characterized biologically, chemically, and physically, so that information about its biosynthesis, if not already available, may reasonably be anticipated. It should not occur in the egg but

should have a developmental history such that its emergence is one consequence of differentiation. Finally, it should participate detectably in distinctive structures or functions in later embryonic or adult stages.

Although a few macromolecules fulfil one or more of these requirements (e.g., nucleic acid, myosin, cholinesterase, fertilizin), none of them is ideal, as several embryological investigations attest. There is one substance, collagen, which does satisfy the criteria listed above but which, despite long and extensive study by a variety of methods, has not received the attention it deserves from modern embryologists. Collagen is one of the best-known biologically important macromolecules; recent physical and chemical studies have provided abundant information about its shape, size, and structure.[1-3] The value of collagen for analyses of morphogenesis at the macromolecular and macromolecular aggregate levels is well established.[4-6] The biosynthesis of collagen has been studied during the healing of adult skin wounds (for references, see Gould and Woessner[7]) and in tissue cultures.[8-10]

The developmental appearance of collagen should be readily traceable because, first, it gives a distinctive X-ray diffraction pattern; second, it is apparently unique among animal proteins in containing large amounts of hydroxyproline; and, third, it normally forms cross-banded fibrils with an axial period averaging about 640 A and a detailed intraperiod fine structure. Despite these features, there is little information about the earliest origins of collagen in the embryo. Neuman[11] found about 1 μg of hydroxyproline in the 5-day chick embryo and by day 8, when Neuman's data show approximately 100 μg hydroxyproline/embryo, Jackson[12] was able to detect collagen fibrils in electron micrographs of the metatarsal tendon. In reporting a chromatographic analysis of amino acids in eggs and embryos of the frog, Holtfreter *et al.*[13] implied in the description of their Figure 1 that hydroxyproline is already present in the egg but gave no further attention to this possibility.

The results described below represent the initial phase of an investigation undertaken to determine whether collagen is, in fact, suitable for a developmental study of the kind suggested here. Answers were sought to two questions: When during frog development does hydroxyproline appear? and What is the subsequent history of its accumulation?

METHODS

Embryos and larvae of the frog, *Rana pipiens*, were raised from eggs obtained following implantation of pituitary glands into mature females. A series of stages was studied, ranging from the unfertilized egg (Stage 1; Shumway[14]) to larvae which had been feeding for up to 3 weeks at room temperature (Stage II; Taylor and Kollros[15]).

Five to 20 embryos or larvae were analyzed at each stage. Autoclave extracts were prepared and hydrolyzed following the procedure of Gould and Woessner.[7] The autoclave residues were uniformly free of hydroxyproline and will not be considered further. Aliquots of the hydrolyzates, representing from one-eighth to one-half the embryos in the original sample, were tested for hydroxyproline by the colorimetric method of Neuman and Logan[16] as modified by Martin and Axelrod.[17] The test proved suitable for detecting down to about 0.5 μg hydroxyproline/ml. of aliquot analyzed if optical density was measured with a spectrophotometer (Beck-

man, Model DU) rather than a colorimeter, as recommended in the original procedure. Immediately following the determination of absorbance at 560 mμ, partial (520–580 mμ) absorption spectra of the test solutions were measured. The former values permitted quantitative estimation of the hydroxyproline content. Because the pink color fades about 1–2 per cent per minute, the absorption spectra were not quantitatively useful. They were invaluable, however, for qualitative information, especially in the case of solutions containing less than 1 μg hydroxyproline/ml.[10] Aqueous solutions of hydroxyproline (H. M. Chemical Co., Santa Monica, Calif.), previously tested for purity by paper chromatography, were used as standards. A solution of hydrolyzed Eastman Kodak gelatin was included in each test as a control.

TABLE 1

ANALYSES OF SERIES III EMBRYOS

Stage (1)	Dry Weight per Embryo (mg.) (2)	Dry Wt. × 100 / Wet Wt. (Per Cent) (3)	Autoclave Extract; Per Cent of Dry Weight (4)	Hydroxyproline per Embryo (μg.)* (5)
1	1.20	12.6†	33.3	0.16
9	1.19	22.6	31.1	0.16
12	1.18	18.0	32.2	0.16
16	1.10	25.3‡	33.7	0.14
18	1.13	22.7	30.9	0.14
21	1.07	17.6	30.9	0.26
22	1.09	15.8	30.3	0.32
23	1.11	10.9	31.5	0.48
24	1.13	10.7	32.7	0.74
25	1.04	7.1	37.5	1.36
25^{+2}	1.09	5.1	56.8	2.42
25^{+4}	1.22	4.3	54.1	3.84
25^{+7}	1.12	3.8	54.5	3.32
25^{+11}	0.99	3.7	49.5	5.12

† Some jelly included in wet weight thus reducing the apparent % dry weight of the egg.

‡ Chorions removed from this and subsequent stages thus eliminating pervitelline fluid from wet weights and increasing the apparent % dry weight.

* Stages up to 21 probably not greater than zero; see text.

Two-dimensional chromatograms of selected stages were run on Whatman No. 1 paper. Aliquots of the hydrolyzates representing two or three embryos were placed on the paper. *N*-butyl alcohol:acetic acid:water (4:1:5) and phenol:*o*-cresol (1:1, water-saturated) mixtures were used as solvents. The length of each run was approximately 30 cm. Spots were developed with ninhydrin (reagent according to Brüggemann and Drepper[18]) or with isatin and Ehrlich's reagent (as recommended by Jepson and Smith[19]).

Continuous preparative paper electrophoresis ("Elphor Va"[20]) was used to separate acid, basic, and neutral fractions of one hydrolyzate. The buffer system was pyridine-acetic acid at pH 3.9. A potential difference of 290 volts was applied across the paper with a current of 15 mA, and an aqueous solution of the sample was delivered to the paper at 0.07 ml/hr. The separation was carried out at 12° C. for 20 hours. Five fractions were collected and analyzed with the Neuman-Logan[16] test.

OBSERVATIONS

Four series of embryos were analyzed. Mutually consistent results were ob-

tained in the four series, but, since Series I and II were mainly exploratory, only the last two will be considered here.

Colorimetric Analyses.—The embryos of Series III developed from the eggs of a single female. After reaching Stage 25 (Shumway[14]) 13 days after fertilization, they were maintained on boiled lettuce and were at Stage II (Taylor and Kollros[15]) 11 days later. Since the stages analyzed after Stage 25 were more closely spaced than those of the Taylor and Kollros series, they are designated Stage 25^{+1}, 25^{+2}, etc., the superscripts indicating days at room temperature after the onset of feeding (cf. Table 1).

Twenty embryos or larvae at each of fourteen stages were studied (Table 1). The data show the expected constancy of fat-free dry weights (col. 2) and steady

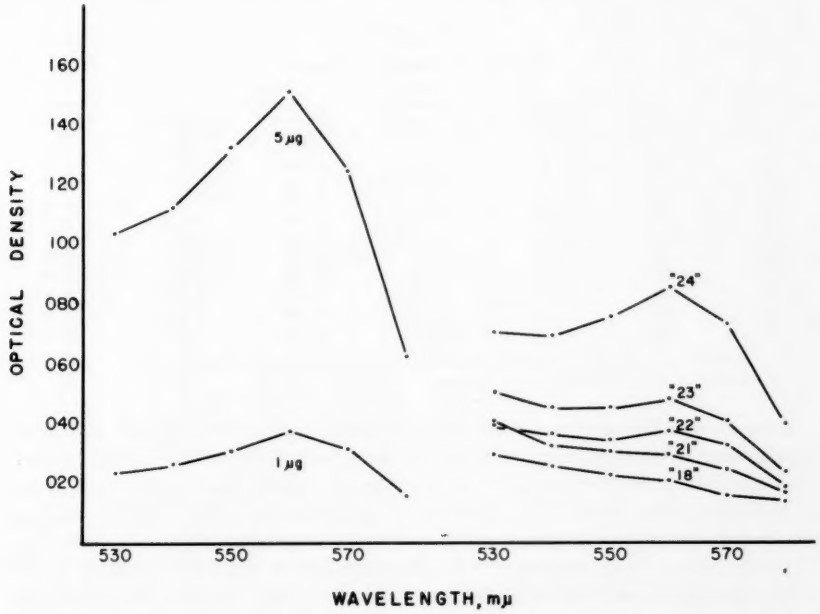

FIG. 1.—Partial absorption spectra (*left*) of aqueous hydroxyproline solutions and (*right*) of hydrolyzates of *Rana pipiens* embryos (Shumway Stages 18–24; 5 embryos/ml).

increase of wet weights (col. 3; cf. Needham[21], pp. 824, 883 ff.). Through Stage 25, there is a similar constancy in the weight of autoclave-extractable fractions; roughly one-third of the dry weight is extracted. Thereafter, there is an abrupt increase in this fraction, and within 2 days (Stage 25^{+2}) over half the dry weight becomes extractable (col. 4). Although some of this increase in autoclave-extractable material may be attributed to recently synthesized collagen, the bulk of it is not collagen, as results presented below will show. Presumably, the increase is due largely to other water-soluble proteins emerging in the embryo at this time.

Hydrolyzed samples of all the stages listed in Table 1 were analyzed colorimetrically for hydroxyproline. The most relevant results are summarized in

Figure 1. This figure shows seven partial absorption spectra, two (*left*) of aqueous hydroxyproline solutions (1 and 5 μg/ml, respectively) and the rest (*right*) of acid hydrolyzates of five embryonic stages. In each case, the spectrum of the 0 μg hydroxyproline standard coincides with the base line. The spectra of the standard solutions show the usual peak near 560 mμ and indicate that 1 μg hydroxyproline/ml can readily be detected. The spectra of the hydrolyzates may be characterized as follows. Two of them (Stages 18 and 21) fall steadily between 530 and 580 mμ and show no peak at 560 mμ. (All the spectra of still younger stages, back to the unfertilized egg, were essentially identical with the Stage 18 curve.) The spectra for the three older stages show progressively larger peaks at 560 mμ and, except for their relatively high densities below 550 mμ, due to an as yet undetermined component, are similar to those of aqueous hydroxyproline.

TABLE 2

ANALYSES OF SERIES IV EMBRYOS

Stage (1)	Age (Hours)* (2)	Dry Weight per Embryo (mg.) (3)	Autoclave Extract; Per Cent of Dry Weight (4)	Hydroxyproline per Embryo (μg.) Aqueous Standard (5)	Hydroxyproline per Embryo (μg.) Hydrolyzate Standard (6)	Collagen† per Embryo (μg.) (7)
18	96	1.20	35.8	0.14	0.0	0.0
21$^+$	172	1.17	35.0	0.28	0.09	0.9
22$^-$	182	1.18	31.4	0.38	0.38	3.8
23$^=$	204	1.22	33.6	0.42	0.47	4.7
23$^-$	204	1.18	33.9	0.42	0.47	4.7
23$^+$	220	1.19	38.7	0.56	0.80	8.0
24$^-$	230	1.05	35.2	0.56	0.80	8.0
24	240	1.36	31.6	0.61	0.89	8.9
25$^-$	260	1.18	42.4	0.80	1.13	11.3
25	284	1.18	45.8	0.94	1.36	13.6

* Estimated for 18° C. from Shumway's tables (*Anat. Rec.*, *78*, 139–47, 1940).

† Column 6 × 10; see text.

Even though the spectra for the two youngest stages show no peaks and hence demonstrate that no hydroxyproline can be detected by this method at these stages, their absolute density values lie well above zero; clearly, some component of the hydrolyzates other than hydroxyproline is contributing to the density readings (cf. Hulliger *et al.*[10]). Although tyrosine is know to produce about 1–1.5 per cent as much color as hydroxyproline in the Neuman-Logan test,[16] it can hardly be the substance mainly responsible for the absorbance here, since its absorption spectrum includes a peak near 560 mμ.[22] In any case, this observation demonstrates that it is quantitatively misleading to use aqueous hydroxyproline solutions as standards against which to compare the hydrolyzates. (It would also be qualitatively misleading to rely on densities at a single wave length.) Hydrolyzates of Stage 18 or younger embryos, to which varying amounts of hydroxyproline had been added, would provide a more valid source of standard curves. The use of such standards is described below but, first, attention is directed to Table 1, column 5, which lists the hydroxyproline contents per embryo based on the admittedly rough values of this series. That is, the values for the early stages up to Stage 21 are probably not greater than zero, while those for older stages can be regarded only as approximations.

In Series IV, both aqueous hydroxyproline solutions and hydrolyzates of Stage 18 embryos, to which known amounts of hydroxyproline had been added, were

used as standards. Because Series III had focused attention on Stages 18–25 as the developmental period when hydroxyproline was appearing, the analyses of Series IV were confined to these stages. At each of the stages listed in Table 2, ten of the embryos from a single clutch of eggs were prepared for analysis.

The standard curves for this series are plotted in Figure 2; they show the usual near-linear relation between hydroxyproline concentration and optical density at

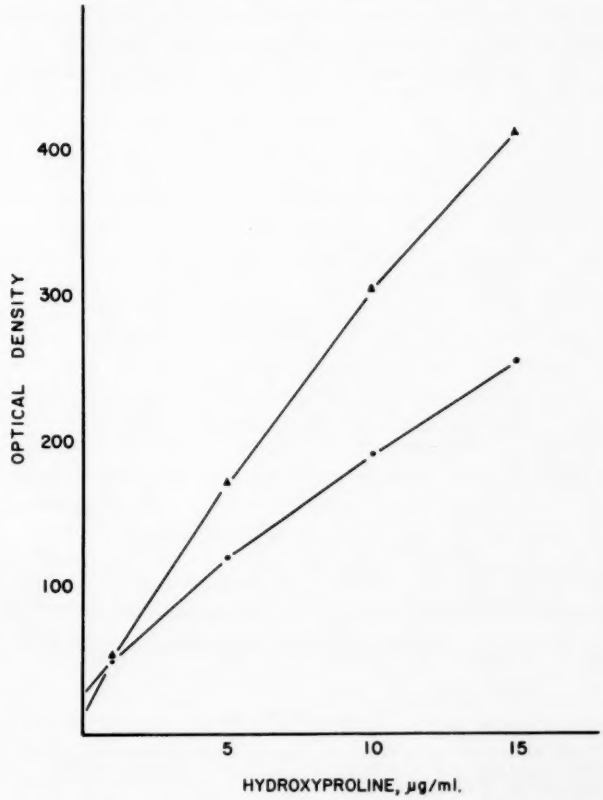

FIG. 2.—Calibration lines for Series IV, showing optical density at 560 mμ as a function of hydroxyproline concentration. *Triangles:* aqueous hydroxyproline solutions; *solid circles:* hydroxyproline added to Stage 18 hydrolyzate ($2^1/_8$ embryos/ml).

560 mμ. The slopes of the two lines reveal, however, that a given amount of hydroxyproline added to a hydrolyzate of Stage 18 embryos produces much less color (*solid circles*) than the same amount of imino acid in pure solution (*triangles*). In demonstrating that the hydrolyzate contains some substance which interferes with the development of color, these data give further support to the importance of using hydrolyzates as standard solutions. No exhaustive attempts have been made to identify the interfering substance. Apparently, none of the amino acids

in gelatin is responsible; gelatin hydrolyzates included routinely in all tests gave values for hydroxyproline close to those generally accepted. Similarly, amino acids which are either absent from or in very low concentration in collagen but which would be expected in hydrolyzates of other proteins—cystine, histidine, phenylalanine, and tyrosine—did not interfere with the hydroxyproline test when they were added either singly or together to gelatin hydrolyzates in amounts up to 25 μg/ml (however, cf. Martin and Axelrod[17]).

Partial absorption spectra of 1 and 5 μg standard solutions and of embryo hydrolyzates measured against hydroxyproline-hydrolyzate standards are shown in Figure 3. The Stage 18 spectrum, which is based on different embryos than those

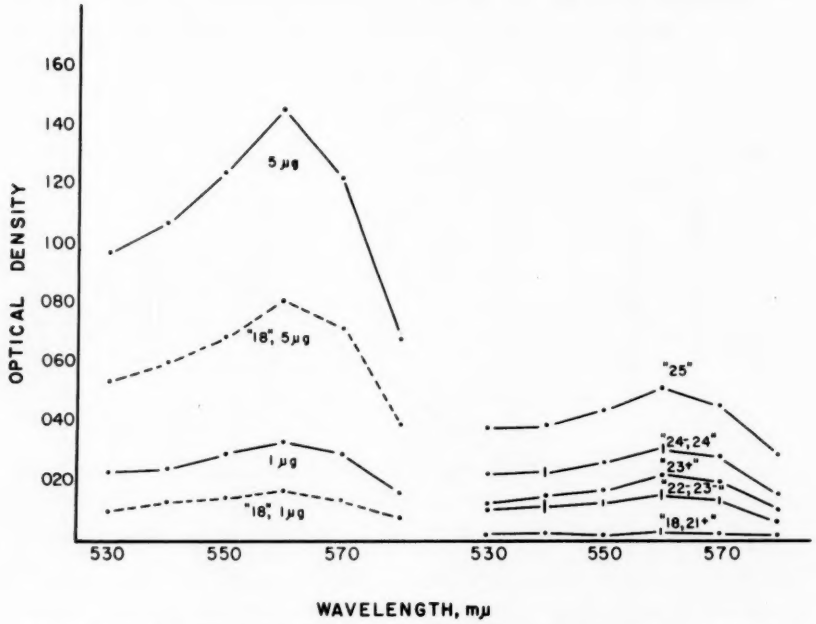

FIG. 3.—Partial absorption spectra (*left*) of aqueous hydroxyproline (*solid lines*) and hydrolyzate-hydroxyproline (*dotted lines*) standards, and (*right*) of hydrolyzates of *Rana pipiens* embryos (Shumway Stages 18–25; $2^1/_2$ embryos/ml).

used for the standard solutions, shows no significant absorption; nor does the Stage 21 spectrum, which is plotted with it. (In plots of more than one stage, vertical bars are used when the densities differ by more than 0.002.) Beginning at Stage 22^-, the spectra begin to show clear absorption peaks at 560 mμ, which become progressively higher with increasingly older embryos.

The amounts of hydroxyproline per embryo, calculated from both aqueous and hydrolyzate standard curves (Fig. 2), are given in Table 2, columns 5 and 6. The use of the hydrolyzate standard yields lower values for the younger stages and higher values for the older. In view of the arguments advanced above, the values based on this standard are accepted as the more reliable. They were therefore

used to calculate the amount of collagen per embryo (col. 7). (Collagen content equals hydroxyproline content × 10; amphibian collagen has a hydroxyproline content somewhat lower than that of mammals, some 10 per cent as compared to 12–13 per cent).[23,24]

Chromatographic and Electrophoretic Analyses.—Two other procedures, namely, paper chromatography and continuous paper electrophoresis, were employed in an effort to provide additional evidence that the substance giving a pink color in the Newman-Logan test was, in fact, hydroxyproline. The paper chromatograms of embryo hydrolyzates all showed an array of well-separated chromagens typical of a complex mixture of amino acids and demonstrated that hydrolysis had been complete. All the expected amino acids except hydroxyproline, but including proline, were detected. This was true of all stages analyzed up to and including Stage 25^{+7} (Stage I of Taylor and Kollros[15]). In the latter case the chromatographed sample contained the amino acids from an extract of two embryos of Series III and should have included some 6–7 μg of hydroxyproline, judging from the Neuman-Logan test applied to the same material (Table 1). Employing isatin and dimethylaminobenzaldehyde as color reagents, 2 μg of pure hydroxyproline spotted directly on the paper could be detected easily. Yet no hydroxyproline chromagen could be demonstrated with these reagents on the chromatogram of the Stage 25^{+7} hydrolyzate. The use of ninhydrin, alone or followed by treatment with dilute alkali and acid as recommended by Pasieka and Morgan,[25] also failed to develop a visible hydroxyproline spot. With the latter method, moreover, 5 μg of hydroxyproline added to the hydrolyzate before chromatographing could not be detected.

Although the chromatographic results cast some doubt on the nature of the substance revealed in this study by the Neuman-Logan test, the evidence obtained from continuous paper electrophoresis increases the probability that it is hydroxyproline. A hydrolyzate of twenty Stage 25^{+4} embryos was separated into four strongly ninhydrin-positive fractions presumably containing, respectively, aspartic acid, glutamic acid, neutral amino acids, and basic amino acids. A fifth fraction, quantitatively small and showing as a faint yellow line on the cathode side of the paper upon treatment with ninhydrin, was also collected.

When these five fractions were subjected to the Neuman-Logan test, only the neutral amino acid fraction developed the pink color characteristic of hydroxyproline. It absorbed strongly, with a sharp peak at 560 mμ, where its optical density was 0.349. The absorption spectra of the four remaining fractions showed no peaks between 530 and 580 mμ. Between these wave lengths, the basic amino acid fraction had a uniformly higher optical density (0.054 at 560 mμ) than the other three fractions (0.009–0.017 at 560 mμ). Apparently, the basic amino acid fraction contains the substance responsible in embryo hydrolyzates for non-specific absorption. No analysis was made of the capacity of this fraction to interfere with the recovery of added hydroxyproline, but it should be considered in any future effort to identify the substances responsible for these effects. Basic amino acids, however, are apparently not involved. Substantial amounts of lysine and arginine are present in, and histidine may be added to, gelatin hydrolyzates without appreciably influencing the values for hydroxyproline obtained in the Neuman-Logan test (see also Neuman and Logan[24] and Grunbaum and Glick[22]).

DISCUSSION

The first appearance and subsequent history of hydroxyproline during embryonic and early larval development of the frog can be established with considerable precision, as the foregoing results demonstrate. The data indicate that this imino acid emerges in *Rana pipiens* between Stages 21 and 22 and increases linearly thereafter through Stage II.

As for the amounts of collagen which are represented by the hydroxyproline values reported, there is an element of uncertainty. Recent studies[23,26] indicate that the skin collagen of one amphibian species (*Xenopus laevis*) is, as might be expected, more like that of fish than of mammals. If Leach's[23] value of 10 per cent hydroxyproline for *Xenopus* collagen holds for *R. pipiens* embryos, then some 13–14 μg of collagen per embryo undergo at least the final stages of synthesis during the 5 days of development between Stages 21 and 25; collagen then represents some 1.2 per cent of the fat-free dry weight of the embryo (calculated from data in Table 2), a value similar to that obtained by Neuman[11] for the 8-day chick embryo (which, however, contains well over fifty times as much collagen as a Stage 25 frog embryo).

The data offer no hints as to the specific nature of the collagen being measured. Thus it is not possible to make any commitment about precursor or definitive collagen molecules. On the basis of their solubility properties, at least three forms of collagen are believed to exist: acid-soluble, neutral-salt–soluble, and insoluble unless converted to gelatin. The present tendency is to regard neutral-salt–soluble collagen as a precursor form which can be transformed into the other two types (for references see Schmitt *et al.*;[5] Jackson[27]). However, each of the three forms contains hydroxyproline, and any or all could occur in the frog embryos described here.

In view of the answers obtained in the present study as to when collagen first appears during embryonic life, questions about where this substance is first detectable become pressing. Preliminary findings indicate that much, though not all, of the hydroxyproline occurs in the basement membrane of the epidermis, a result which might have been predicted from its fine structure.[6] The developmental history of the basement membrane is now being investigated both chemically and electron-microscopically.

SUMMARY

Using the hydroxyproline content as a means of detecting collagen, the early history of this fibrous protein has been traced in the frog embryo. Hydroxyproline first appears between Stages 21 and 22, the latter containing about 0.4 μg/embryo, and increases linearly at least through Stage II, when some 5 μg/embryo are present. These hydroxyproline values correspond to 4 and 50 μg of collagen, which thus comprises some 0.3 and 5 per cent, respectively, of the fat-free dry weights. The results suggest that collagen is particularly suitable for a study of embryonic chemodifferentiation.

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*AMINO ACID INCORPORATION AND THE REVERSION OF ITS INITIAL PHASE WITH CELL-FREE TETRAHYMENA PREPARATIONS**

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Cell-free preparations of various biological systems present a rather uniform pattern of amino acid incorporation. However, often the different systems seem to be more or less appropriate for studying a particular step in the sequence of reactions from initial activation[1] to final incorporation into protein. In the present study, *Tetrahymena pyriformis* was chosen as a test organism. This protozoön yields subcellular fractions analogous to those obtained with mammalian tissue homogenates. The *Tetrahymena* system was found to be particularly suitable for the demonstration of a reversibility of transfer of activated amino acid to the soluble ribonucleic acid fraction.

FRACTIONATION OF HOMOGENATES

Tetrahymena pyriformis "W" was kindly supplied to us by Dr. G. W. Kidder. It was grown in a shallow layer of 2 per cent Difco proteose at 26° for 40 hours. The yield was approximately 0.7 gm. of dry weight per liter. The organisms were collected by decantation and centrifugation at 400 *g* for 5 minutes, suspended in one-fourth of the original volume of distilled water, and washed twice in the centrifuge. The washed residue was suspended in 50 ml. of 0.4 *M* sucrose solution, containing 0.1 *M* Tris[2] buffer, pH 7.5, and 0.005 *M* potassium chloride, and the suspension was left on ice for 10 minutes to lyse partially. This preparation separated, on centrifugation for 15 minutes at 18,000 *g*, into a well-packed bottom layer, a fluffy viscose intermediate layer, with a clear supernatant on top. The supernate and the intermediate layer were decanted and discarded, the pellet was re-homogenized with a Teflon homogenizer in 10 ml. of 0.25 sucrose to complete cellular disintegration, making the preparation suitable for differential centrifugation. Cell debris and nuclei were spun off at 1,500 *g* and discarded. For further fractionation, the sucrose concentration was raised to 0.35 *M*. On centrifugation at 12,000 *g* for 15 minutes a mitochondrial fraction was sedimented. The turbid supernate of this was further separated by centrifugation at 105,000 *g* for 90 minutes, into a translucent microsomal pellet and a supernatant soluble fraction.

ASSAY FOR INCORPORATION

For assaying amino acid incorporation, an incubation mixture was used, containing, in a final volume of 1 ml., the following ingredients: 100 μmoles Tris buffer, pH 7.6; 6 μmoles magnesium chloride; 0.3 μmoles DL-leucine-1-C^{14}; and an ATP-generating system consisting of 1 μmole ATP, 5 μmoles lithium carbamyl phosphate with 0.01 ml. of a dialyzed sonic extract of *Streptococcus faecalis*, which served as carbamyl phosphokinase.[3]

TABLE 1*

INCORPORATION OF LEUCINE† INTO SUBCELLULAR FRACTIONS OF *Tetrahymena*

Fraction	C.p.m. per Mg. Protein
Mitochondria	187
Microsomes	258
Soluble fraction (S.F.)	8
Microsome + S.F.	684
Mitochondria + S.F.	135

* Standard reaction mixture as described in assay for incorporation; in addition: mitochondria twice washed with 0.25 *M* sucrose, 5.6 mg. protein; microsomes, 2 mg. protein; S.F., 1.5 mg. protein; incubated for 60 minutes at 25°.

† Radioactive valine, alanine, phenylalanine, and serine gave similar but only about half as much incorporation.

The radioactive leucine was kindly prepared for our use by Dr. Loftfield. It contained 5 μC per μm, which in our Nuclear counter is equivalent to 8×10^6 counts per μm. With only the L-component reactive, this corresponds to the addition in 0.3 μmoles of DL-leucine of roughly 1×10^6 counts per test. The counting technique used was the same as described by Weiss *et al.*[1]

To determine protein-bound amino acids, the TCA precipitate was washed with

hot TCA according to Schneider[4] and with ether-alcohol to remove a lipid material. The non-protein (nucleotide-bound) amino acids were determined as the difference between cold and hot TCA-washed residue.

Incorporation into Microsome-Protein.—The activities of various fractions and combinations are shown in Table 1. It appears that, as with liver and other systems, the highest rate of incorporation was obtained with a mixture of microsomal and supernatant fractions. The mitochondrial fraction gave a significant amino acid incorporation, which, however, seemed not to be increased by the addition of the soluble fraction. The soluble fraction alone showed no uptake of amino acids into *protein*.

As shown in Table 2, the stimulatory effect of the homologous supernate on the incorporation into *Tetrahymena* microsomes could be duplicated to a large extent by a supernate derived from a liver homogenate. Likewise, liver microsomes were found to respond strongly to the addition of *Tetrahymena* supernate, demonstrating the interchangeability between protozoan and mammalian preparations.

TABLE 2*

INCORPORATION OF RADIOACTIVE LEUCINE INTO VARIOUS COMBINATIONS

	Fractions	Total c.p.m. "Protein-bound"†	Total c.p.m. "Nucleotide-bound"†
1*a*	Tetrahymena microsomes	650	...
	Tetrahymena microsomes + *Tetrahymena* supernate	1,780	...
	Tetrahymena microsomes + liver supernate	1,235	...
1*b*	Liver microsomes	794	...
	Liver microsomes + *Tetrahymena* supernate	3,115	...
	Liver microsomes + liver supernate	4,276	...
2	Supernate *Tetrahymena*	4	946

* Incubation analogous to that of Table 1.
† "Protein-bound" refers to *hot* TCA-treated; "nucleotide-bound" refers to *cold* TCA-washed residue.

TABLE 3*

REVERSAL OF LEUCINE INCORPORATION INTO NUCLEOTIDE-BOUND FRACTION OF SUPERNATE

No.	Added after Initial 10 Min. Incubation	Total c.p.m. after 1st 10 Min.	Total c.p.m. after 2d 10 Min.
1	None	1,120	1,138
	KF + AMP	..	982
	KF + PP	..	294
	KF + PP + AMP	..	68
2	None	1,240	1,175
	AMP	..	1,260
	PP	..	815
	PP + AMP	..	328

* Incubation analogous to that described in Table 1, but only supernate was used. The compounds added after 10-min. initial incubation were present at the following levels: KF, 10 μmoles; AMP, 5 μmoles; PP, 5 μmoles. All counts refer to residue precipitated and washed with *cold* TCA and ether-ethanol ATP, 2 μmoles instead of regeneration system.

Attachment to Soluble Polynucleotide.—In the second experiment presented in Table 2, where the sediment was extracted with cold TCA only, an incorporation was obtained with the non-particulate supernate alone. The amino acid incorporated under these conditions, however, in contrast to the microsomal, protein-bound acids, was released by treatment with hot TCA, with RNA-ase, or with dilute alkali. This is interpreted to indicate formation of polynucleotide-bound amino acid in the supernate, in a similar manner to that described by Hoagland

et al.[5] and by Weiss *et al.*[1] for liver and pancreas, respectively. The order of magnitude of this incorporation was similar to that found in the microsomal protein.

Reversion.—This incorporation into the RNA fraction was found, as shown in Table 3, to be reversed by the addition of PP or, more effectively, by a mixture of PP and AMP. Fluoride increased the effect, presumably by inhibition of the rather abundant pyrophosphatase present in the preparation. Without fluoride (cf. No. 2 in Table 3), AMP alone was inactive, while with fluoride it probably showed a slight effect. In contrast to PP, inorganic P was entirely inactive.

COMMENTS

It appears that the protozoön *Tetrahymena* contains an amino acid incorporation system rather similar to that of higher animals. The non-particulate fraction here likewise incorporates amino acids in a linkage which is hot-TCA-, RNA-ase-, and alkali-unstable and is therefore presumably due to linkage with soluble polynucleotide. This incorporation is of a magnitude similar to that in the microsomal protein.

The *Tetrahymena* preparation proved to be rather suitable for showing a reversibility of the initial step, namely, the amino acid attachment to soluble polynucleotide. Such a reversion is obtained by the addition of the PP and is considerably enhanced by PP + AMP. This indicates that the transfer of the activated amino acid, presumably amino acyl adenylate, to the polynucleotide may be reversed on addition of both reaction products. The pronounced effect of AMP to reverse incorporation gives welcome support to a two-step reaction (cf. also Holley[6]) to be formulated as follows:

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{amino acyl-AMP} + \text{PP}$$
$$\text{Amino acyl-AMP} + \text{polynucleotide} \rightleftharpoons \text{amino acyl-polynucleotide} + \text{AMP}$$

The inability of AMP alone to reverse appreciably may mean that in this sequence the amino acyl adenylate is not a free intermediary. It may also, however simply indicate that the reversal of the second reaction is energetically unfeasible without a simultaneous reversal of the first step, which we know to be quite exergonic backwards. A non-reversal by AMP in this interpretation may indicate the second step to be rather irreversible in *forward* direction and balancing the endergonic initial reaction. We prefer, at present, not to discuss this somewhat foggy situation further, but feel that the present formulation is probably an over-simplification.

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† This work was carried out during the tenure of a Warburg-Magnes fellowship of the Hebrew University, Hadassah Medical School. Present address: Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem.

[1] S. B. Weiss, G. Acs, and F. Lipmann, These PROCEEDINGS (in press).

[2] The following abbreviations have been used: AMP, adenosine monophosphate; ATP, adenosine triphosphate; P, phosphate; PP, pyrophosphate; TCA, trichloracetic acid; RNA, ribonucleic acid; Tris, tris (hydroxymethyl) amino methane.

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*SOLUTION OF A PAIR OF INTEGRAL EQUATIONS FROM ELASTOSTATICS**

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Introduction.—In the theory of elastostatics, certain mixed boundary-value problems lead to dual integral equations. For instance, one may consider the elastostatic boundary-value problem presented by specifying zero shear stress over the entire plane boundary of an elastic half-space, while the normal component of stress vanishes on this surface exterior to a finite strip and the normal component of displacement is specified within this strip. This two-dimensional problem is formulated conveniently in terms of the type of dual integral equations considered in this paper. For the details of the formulation the reader is referred to the work of Sneddon.[1]

If the normal component of displacement is decomposed into its even part, $g(x)$, and its odd part, $h(x)$, the resulting integral equations are

$$\int_0^\infty \xi^{-1} P(\xi) \cos(\xi x) d\xi = g(x), \qquad 0 \le x < a, \quad (a)$$

$$\int_0^\infty P(\xi) \cos(\xi x) d\xi = 0, \qquad a < x \le \infty, \quad (b) \tag{1}$$

and

$$\int_0^\infty \xi^{-1} Q(\xi) \sin(\xi x) d\xi = h(x), \qquad 0 \le x < a, \quad (a)$$

$$\int_0^\infty Q(\xi) \sin(\xi x) d\xi = 0, \qquad a < x \le \infty. \quad (b) \tag{2}$$

The systems of equations (1) and (2) have been considered by Sneddon,[2] who transformed the kernel functions into Bessel functions of order one-half. The resulting dual integral equations bear a formal resemblance to the equations solved by Busbridge.[3] However, the restrictions on the solution of Busbridge are violated by the equations (1) and (2), and Sneddon's use of this solution is not justified.

Solution of the Dual Integral Equations.—Since the superposition theorem holds for elastostatics, it is possible to decompose the normal displacement prescribed on the surface $-a < x < a$, $z = 0$, into an even and an odd function of x, as above. It is possible to decompose further both the even and odd functions $g(x)$ and $h(x)$ and solve a number of separate sets of dual integral equations. To the term-by-term decomposition of $g(x)$ and $h(x)$, there will correspond a term-by-term decomposition of the unknown functions $P(\xi)$ and $Q(\xi)$. After solving the separate problems, one may recombine the solutions to obtain the solution of the composite problem.

We shall assume that $g(x)$ and $h(x)$ are functions which may be represented by the Fourier series:

$$g(x) = \sum_{n=0}^{\infty} C_n \cos(n\pi x/a), \tag{3}$$

$$h(x) = \sum_{n=1}^{\infty} D_n \sin(n\pi x/a). \tag{4}$$

Furthermore, if we define the angle variable

$$\varphi = \text{arc sin}(x/a)$$

and use the familiar expansions[4]

$$\cos(n\pi \sin\varphi) = J_0(n\pi) + 2\sum_{q=1}^{\infty} J_{2q}(n\pi)\cos[2q \text{ arc sin}(x/a)]$$

$$\sin(n\pi \sin\varphi) = 2\sum_{q=0}^{\infty} J_{2q+1}(n\pi)\sin[(2q+1)\arcsin(x/a)],$$

we may write

$$g(x) = \sum_{n=0}^{\infty}\sum_{q=0}^{\infty} \epsilon_q C_n J_{2q}(n\pi)\cos[2q\arcsin(x/a)], \tag{5}$$

$$\epsilon_q = 1, \quad q = 0; \qquad \epsilon_q = 2, \quad q > 0,$$

$$h(x) = 2\sum_{n=1}^{\infty}\sum_{q=0}^{\infty} D_n J_{2q+1}(n\pi)\sin[(2q+1)\arcsin(x/a)]. \tag{6}$$

We write equations (1) in the form

$$\int_0^{\infty} \xi^{-1} P_{nq}(\xi)\cos(\xi x)d\xi = \epsilon_q C_n J_{2q}(n\pi)\cos[2q\arcsin(x/a)], \quad 0 \leqslant x < a, \quad (a)$$

$$\int_0^{\infty} P_{nq}(\xi)\cos(\xi x)d\xi = 0, \qquad a < x \leqslant \infty, \quad (b) \tag{7}$$

employing the superposition principle. The solution may be obtained by inspection if we consider the discontinuous integrals of Weber,[5]

$$a\int_0^{\infty} J_1(a\xi)\cos(\xi x)d\xi = 1, \qquad 0 \leq x < a, \quad (a)$$

$$a\int_0^{\infty} \xi J_1(a\xi)\cos(\xi x)d\xi = 0, \qquad a < x \leq \infty, \quad (b) \tag{8}$$

$$\int_0^{\infty} \xi^{-1} J_{2q}(a\xi)\cos(\xi x)d\xi = (2q)^{-1}\cos[2q\arcsin(x/a)], \qquad 0 \leq x < a \quad (c)$$

$$\int_0^{\infty} J_{2q}(a\xi)\cos(\xi x)d\xi = 0, \qquad a < x \leq \infty. \quad (d)$$

From equations (8a) and (8b) we obtain $P_{no}(\xi)$, and from equations (8c) and (8d) we obtain $P_{nq}(\xi)$ for positive integral values of q. The solution of the composite problem becomes

$$P(\xi) = a\xi J_1(a\xi)\sum_{n=0}^{\infty} C_n J_0(n\pi) + 4\sum_{n=0}^{\infty}\sum_{q=1}^{\infty} qC_n J_{2q}(n\pi)J_{2q}(a\xi). \tag{9}$$

The solution of equations (2) is obtained by an identical process, the details of which we omit. The resulting solution is

$$Q(\xi) = 2\sum_{n=0}^{\infty}\sum_{q=1}^{\infty}(2q+1)D_n J_{q+1}(n\pi)J_{2q+1}(a\xi). \tag{10}$$

The Convergence of the Series Solutions.—We consider, first, the series solution given by equation (9). The series

$$\sum_{n=0}^{\infty} C_n J_0(n\pi)$$

is a special case of the Schlomilch series,[6]

$$f(y) = \frac{C_0}{2} + \sum_{n=1}^{\infty} C_n J_0(ny), \tag{11}$$

which is known to converge in the half-open interval $0 < y \leq \pi$, provided that the coefficients C_n obey the order condition,

$$C_n \sim n^{-\alpha}, \qquad \alpha \geqslant 1/2.$$

Since the coefficients C_n are Fourier expansion coefficients, they obey this condition.

We consider the series

$$T(n, \xi) = \sum_{q=1}^{\infty} 4q\,|J_{2q}(n\pi)|\,|J_{2q}(a\xi)\,| \tag{12}$$

obtained from the second part of equation (9) by making all the terms positive. We fix the values of n and ξ at the numbers N and Ξ, which may be made arbitrarily large. Let Q be the first integer which is larger than the greater of the numbers $N\pi$ and $(a\Xi)$. Then we may write

$$T(N, \Xi) = \sum_{q=1}^{Q-1} 4q\,|J_{2q}(N\pi)|\,|J_{2q}(a\Xi)\,| + \sum_{q=Q}^{\infty} 4q\,|J_{2q}(N\pi)|\,|J_{2q}(a\Xi)\,|. \tag{13}$$

The first partial sum is finite, since each term of equation (12) is bounded for any arbitrarily large or small value of N and Ξ. We therefore shall establish the convergence of the remainder sum,

$$R(N, \Xi) = \sum_{q=Q}^{\infty} u_q(N, \Xi) = \sum_{q=Q}^{\infty} 4q\,|J_{2q}(N\pi)|\,|J_{2q}(a\Xi)\,|. \tag{14}$$

Using the ratio test for sequences of positive terms, we examine

$$\lim_{q\to\infty}\{u_{q+1}/u_q\}.$$

If we set

$$N\pi = q\epsilon, \qquad 0 < \epsilon < 1; \qquad a\,\Xi = q\delta, \qquad 0 < \delta < 1,$$

and use the inequality[7]

$$J_{2q}(q\beta) \leq (1-\beta^2)^{-1/4}\,(4\pi q)^{-1/2}\exp\{-2qF(0, \beta)\}, \qquad \beta = \epsilon \text{ or } \delta,$$

the ratio becomes

$$\lim_{q\to\infty}\{u_{q+1}/u_q\} \leq \lim_{q\to\infty} |e^{-2q[F(0,\epsilon)+F(0,\delta)]}. \tag{15}$$

Since the quantities $F(0, \epsilon)$ and $F(0, \delta)$ are greater than zero,[8] we see that the

ratio test indicates the absolute convergence of the series solution (9) for any arbitrarily large closed interval of the variable ξ. Furthermore, the series formed by multiplying each term of equation (9) by the quantities $\cos(x\xi)$ or $\xi^{-1}\cos(x\xi)$ retain the absolutely convergent property.

The convergence of the series solution given by equation (10) may be examined in like fashion; and this series and the series formed by multiplying each term of equation (10) by the quantities $\sin(x\xi)$ and $\xi^{-1}\sin(x\xi)$ are found to be absolutely convergent for arbitrarily large intervals of the variable ξ.

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[6] *Ibid.*, p. 621.

[7] *Ibid.*, p. 255.

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*CLASS NUMBER OF A DEFINITE QUATERNION WITH PRIME DISCRIMINANT**

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1. *Introduction.*—According to Eichler, normal division algebras over the field of rationals which can have class numbers greater than 1 are definite quaternions, i.e., normal division algebras of fourth order ramified at infinity. He also obtained[1] a formula for the class numbers of definite quaternions, which in the special case of prime discriminants takes the following form:

$$h = {}^1/_3\cdot(1 - (-3/p)) + {}^1/_4\cdot(1 - (-4/p)) + {}^1/_{12}\cdot(p - 1).$$

Here p is the finite prime at which the quaternion is ramified. Now Deuring observed[2] that the class number of a definite quaternion ramified at p is equal to the number of birationally distinct elliptic curves of characteristic p having no points of order p. The absolute invariants of such elliptic curves are called "supersingular." If, therefore, we can count the number of supersingular invariants of characteristic p, we will get a new proof of the Eichler formula. Deuring thought that such a direct computation was *nicht leicht*. In this paper we shall show that a direct computation is actually possible. The crucial point is that the so-called Hasse invariant

satisfies a differential equation of the Gauss-Legendre type. Thus the Eichler formula is proved purely algebraically and, in fact, in a conceptual way. This seems to be a remarkable case, at least at present, where the class number formula is proved algebraically without using Dirichlet's method.

2. *Hasse Invariant.*—We first note that the case $p = 2$, i.e., the case of Hamilton quaternion, can be treated directly. Therefore, we can assume that p is odd. Let A be an elliptic curve, i.e., an Abelian variety of dimension 1, of characteristic p. Then A is isomorphic to a plane elliptic curve defined by the equation $Y^2 = X(1 - X)(\lambda - X)$ with the point at infinity as neutral element. Here the parameter λ is different from 0, 1, and ∞. Deuring calculated[3] the Hasse invariant of this model, and he got

$$A(\lambda) = (-1)^r \sum_{i=0}^{r} \binom{r}{i}^2 \lambda^i,$$

with $r = {}^1/_2 \cdot (p - 1)$. The absolute invariant of the model is given by

$$j = 2^8 \frac{(1 - \lambda(1 - \lambda))^3}{\lambda^2(1 - \lambda)^2}.$$

Now, as we can see, the polynomial $A(\lambda)$ is a solution of the following differential equation:

$$\lambda(1 - \lambda)d^2K/d\lambda^2 + (1 - 2\lambda)dK/d\lambda - {}^1/_4 \cdot K = 0.$$

In the classical case, this is the differential equation for the periods of the elliptic differential of the first kind of the model considered as normal functions of the parameter λ. Moreover, the regular integral around $\lambda = 0$ is given by a hypergeometric series. In the case of characteristic p, the difference between regular and nonregular solutions disappears, because we have $A(1 - \lambda) = (-1)^r A(\lambda)$. Hence the elliptic differential of the first kind has only one period, and that is $A(\lambda)$ up to an arbitrary differential constant. This version of Hasse invariant has not yet been explicitly remarked. At any rate, since r roots of $A(\lambda)$ are different from 0, 1, and ∞, from the differential equation, it follows that the r roots are all simple.

Now consider the correspondence between the affine j-line and the λ-space, which is an affine line minus 0, 1. The Galois group of the equation is known, i.e., the 6 conjugates of λ over j are the following: λ, $1/\lambda$, $1 - \lambda$, $1/(1 - \lambda)$, $\lambda/(\lambda - 1)$, $(\lambda - 1)/\lambda$. Hence, if we exclude the case $p = 3$ temporarily, to j different from 0 and 12^3 correspond 6 different values of λ. In case $j = 0$, we get only 2 values of λ, and these are the roots of the equation $1 - \lambda + \lambda^2 = 0$. In case $j = 12^3$, we get 3 values of λ, and these are -1, ${}^1/_2$, and 2.

3. *Concluding the Proof.*—We know[4] that the elliptic curve has no points of order p if and only if its Hasse invariant is 0, i.e., if and only if λ is one of the r simple roots of $A(\lambda)$. Let Σ be the set of the corresponding supersingular invariants. In the case $p = 3$ we have $\Sigma = \{0\}$. If we exclude this case, we have the following 4 alternatives: (1) Σ does not contain 0, 12^3; (2) Σ contains 0 but not 12^3; (3) Σ contains 12^3 but not 0; (4) Σ contains 0 and 12^3. Since the number of elements of Σ is the class number h of the definite quaternion ramified at p, we get $6h$, $6(h - 1) + 2$, $6(h - 1) + 3$, $6(h - 2) + 2 + 3$ for r according to the above 4 cases.

Then p is congruent modulo 12 to 1, 5, 7, 11. Therefore, the 4 cases are actually possible, and we get

$$h = \begin{cases} {}^1/_{12}\cdot(p-1) & p \equiv 1 \mod 12 \\ {}^1/_{12}\cdot(p-5)+1 & p \equiv 5 \mod 12 \\ {}^1/_{12}\cdot(p-7)+1 & p \equiv 7 \mod 12 \\ {}^1/_{12}\cdot(p-11)+2 & p \equiv 11 \mod 12. \end{cases}$$

If we remark that the number of supersingular invariants for $p = 3$ is 1, we see that we can express these results by the Eichler formula stated in the introduction.

* This work was partially supported by the National Science Foundation.

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NOTE TO MY PAPER "ON MEMBRANES AND PLATES,"

These Proceedings, **36**, 210–216, 1950

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1. We follow the notation of the paper quoted in the title:[1] D is a given simply connected domain, C its boundary curve; u is the minimizing function of the quotient

$$\frac{\int_D \int (\nabla^2 u)^2 d\sigma}{\int_D \int u^2 d\sigma}, \qquad u = \frac{\partial u}{\partial n} = 0 \text{ on } C; \tag{1}$$

we *assume* that $0 \leqq u \leqq 1$ in D (the part $u \leqq 1$ of the assumption is, of course, immaterial); C_ρ is the set of curves $u = \rho$, $0 \leqq \rho \leqq 1$; $A(\rho) = \pi R^2$ is the area of the domain $\rho \leqq u \leqq 1$;

$$G = |\text{grad } u|, \qquad \int_{C_\rho} G ds = P(\rho), \qquad \int_{C_\rho} G^{-1}(\nabla^2 u)^2 ds = Q(\rho).$$

2. In a recent communication Dr. P. R. Beesack, of McMaster University, has called my attention to the fact that the "symmetrized" function $\bar{u} = f(\rho)$, introduced by the formulas (6_1) and (6_2) of the paper quoted, is *not* eligible for the circular plate $\bar{D}$ of radius R_0, $A(0) = \pi R_0^2$. This is due to the convergence of $A(\rho)$ to 0 as $\rho \to 1$. The argument must be modified as follows.

3. We conclude as in III (4) of the previous paper that

$$[Q(\rho)]^{1/2} |A'(\rho)|^{1/2} \geqq - \int_{C_\rho} G^{-1} \nabla^2 u \, ds;$$

hence

$$\int_\rho^1 [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt \geqq -\int_\rho^1\int_{C_t} G^{-1}\nabla^2 u\,ds\cdot dt =$$

$$-\int_{\rho\leqq u\leqq 1}\int \nabla^2 u\,d\sigma = \int_{C_\rho}\frac{\partial u}{\partial n}ds = P(\rho) \geqq \frac{4\pi A(\rho)}{|A'(\rho)|}; \quad (2)$$

here the normal is directed into the *interior* of the domain $\rho \leqq u \leqq 1$, that is, in the direction of the increasing values of u, along $u = \rho$. The contribution of C_t tends to zero as $t \to 1$; indeed, if $u = 1$ holds at certain isolated points, we have grad $u = 0$, and, in addition, the curve shrinks to a point; should $u = 1$ hold along certain curves, grad $u = 0$ must be used.[2]

4. We define the constant ρ_0, $0 < \rho_0 < 1$, by the condition

$$\int_0^{\rho_0} [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt = \int_{\rho_0}^1 [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt, \quad (3)$$

and now introduce the continuous function

$$g(\rho) = \begin{cases} \dfrac{|A'(\rho)|}{4\pi A(\rho)}\displaystyle\int_0^\rho [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt \text{ for } 0 \leqq \rho \leqq \rho_0, \\ \dfrac{|A'(\rho)|}{4\pi A(\rho)}\displaystyle\int_\rho^1 [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt \text{ for } \rho_0 \leqq \rho \leqq 1. \end{cases} \quad (4)$$

In view of Schwarz's inequality, these integrals exist, since the integrals

$$\int_0^1 Q(t)dt \quad \text{and} \quad \int_0^1 |A'(t)|dt = A(0)$$

are finite. As before, we define

$$f(\rho) = \int_0^\rho g(\rho)d\rho. \quad (5)$$

The function $\bar{u} = f(\rho)$ has a continuous derivative for $0 < \rho < 1$, including $\rho = \rho_0$. Now

$$\lim_{R\to R_0}\bar{u} = f(0) = 0, \qquad \lim_{R\to R_0}\frac{d\bar{u}}{dR} = \lim_{\rho\to 0} f'(\rho)\frac{2\pi R}{A'(\rho)} =$$

$$-\lim_{\rho\to 0}[4\pi A(\rho)]^{-1/2}\int_0^\rho [Q](t)^{1/2}|A'(t)|^{1/2}\,dt = 0.$$

Moreover, $\lim_{R\to 0}\bar{u}$ is finite, since

$$\int_\rho^1 [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt \leqq \left\{\int_\rho^1 Q(t)dt\right\}^{1/2}\left\{\int_\rho^1 |A'(t)|dt\right\}^{1/2}$$

$$\leqq [A(\rho)]^{1/2}\left\{\int_0^1 Q(t)dt\right\}^{1/2}, \quad (6)$$

and the integral

$$\int_\rho^1 \frac{|A'(\rho)|}{4\pi A(\rho)}[A(\rho)]^{1/2}\,d\rho = -\int_0\frac{dA}{4\pi A^{1/2}}$$

is convergent. Finally,

$$\lim_{R\to 0}\frac{d\bar{u}}{dR} = \lim_{R\to 0} f'(\rho)\frac{2\pi R}{A'(\rho)} = -\lim_{R\to 0}[4\pi A(\rho)]^{-1/2}\int_\rho^1 [Q(t)]^{1/2}|A'(t)|^{1/2}\,dt.$$

The latter quantity is, in view of the first step in (6), less than

$$[4\pi A(\rho)]^{-1/2}\,[A(\rho)]^{1/2}\left\{\int_\rho^1 Q(t)dt\right\}^{1/2} = (4\pi)^{-1/2}\left\{\int_\rho^1 Q(t)dt\right\}^{1/2} \to 0$$

as $\rho \to 1$. Thus $\bar{u}$ as a function of $Re^{i\theta} = x + iy$ has continuous first derivatives with respect to x and y, including at $R = 0$; indeed,

$$\frac{\partial \bar{u}}{\partial x} = \frac{d\bar{u}}{dR}\frac{x}{R} \to 0 \text{ as } R \to 0; \text{ similarly } \frac{\partial \bar{u}}{\partial y}.$$

Recapitulating, we find that $\bar{u}$ is admissible for the circular plate (it has a discontinuity in the second derivatives).

5. The remaining argument needs only unessential modifications. We have

$$\nabla^2\bar{u} = \pm\,|A'(\rho)|^{-1/2}\,[Q(\rho)]^{1/2}$$

according as $\rho \lesseqgtr \rho_0$, so that the numerator in (1) does not change when we pass from u to $\bar{u}$, D to $\bar{D}$. In view of (2), we again have $g(\rho) \geqq 1, f(\rho) \geqq \rho$, so that the denominator is increased.

This completes the modified argument. The same reasoning settles the problem of the buckling plate.

6. Finally, it is instructive to discuss briefly the case when D is the circle $r \leqq 1$. Then $\bar{u}(R) = \bar{u}(r)$ coincides with the first eigenfunction $v(hr)$, where

$$v(r) = J_0(r)I_0'(h) - J_0'(h)I_0(r), \qquad h = 3.1962$$

(cf. Pólya-Szegö, *op. cit.*, p. 134). This function is decreasing for $0 \leqq r \leqq h$, $v(0) > 0$, $v(h) = 0$. The relation $\rho = [v(0)]^{-1}v(hr)$ holds.
Now

$$\nabla^2\bar{u} = \frac{h}{r}\frac{d}{dr}(r\,v'(hr)),$$

and, taking the equations $(rJ_0'(r))' + rJ_0 = 0$, $(rI_0'(r))' - rI_0 = 0$ into account, we obtain

$$\frac{1}{r}\frac{d}{dr}(r\,v'(r)) = -J_0(r)I_0'(h) - J_0'(h)I_0(r) = -I_0'(h)I_0(r)\left(\frac{J_0(r)}{I_0(r)} + \frac{J_0(h)}{I_0(h)}\right).$$

Here $-1 < J_0(h)/I_0(h) < 0$. Since $J_0(r)/I_0(r)$ is decreasing for $0 < r < j$ [$j = 2.4048$ is the first positive zero of $J_0(r)$], the latter quantity is negative for $r = 0$ and positive for $r = j < h$. Also $\nabla^2\bar{u}$ changes its sign for a single value $r = r_0$, $0 < r_0 < j$, passing there from negative to positive values. This r_0 corresponds to ρ_0, and in this case $\nabla^2\bar{u}$ vanishes at $\rho = \rho_0$.

[1] Cf. also G. Pólya and G. Szegö, "Isoperimetric Inequalities in Mathematical Physics," *Ann. Math. Studies*, **27**, 235–238, 1951, note F.

[2] Discussing the part $0 \leqq u \leqq \rho$ (cf. *loc. cit.*), we must deal in a similar fashion with the points or lines in the interior of D where $u = 0$.

INVARIANT IMBEDDING, WAVE PROPAGATION, AND THE WKB APPROXIMATION

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1. *Introduction.*—In previous papers,[1] we have presented some applications of the principle of invariant imbedding to radiative-transfer and neutron-diffusion processes. This use of invariance principles was stimulated by the fundamental work of Ambarzumian and Chandrasekhar[2] and strongly influenced by the point-of-regeneration technique of Bellman and Harris[3] and the theory of dynamic programming.[4]

Fundamental for the success of these techniques as applied to the above processes is the ability to consider the over-all physical process as a sequence of local processes. For the case of particles, this is easily done. In this paper we wish to indicate how wave propagation may be considered in these terms. It is rather remarkable that our results will be based upon an algorithm that, in general, can yield divergent series.

Following a provocative paper by Bremmer,[5] our aim is to show that wave propagation can be discussed in terms of reflection and refraction at infinitesimally separated interfaces. We shall prove that the convergence of the Bremmer series can be established under a simple assumption concerning the slowly varying nature of the local wave number.

In addition to the insight to the physical phenomena furnished by these techniques, we are also led to a new method for studying the asymptotic behavior of ordinary and partial differential equations of linear type. For the equation

$$u'' + k^2(x)u = 0, \tag{1.1}$$

the Bremmer series is an extension of the known connection between the principal transmitted, or refracted, wave and the WKB method. We shall obtain a generalization of the WKB method to vector-matrix systems of the form

$$y'' + K^2(x)y = 0, \tag{1.2}$$

where $K(x)$ is a positive definite matrix for $x \geq 0$. This yields a new approach to the study of the asymptotic behavior of the solutions of these equations.[6]

Finally, we shall apply functional equation techniques to the problem of determining the wave reflected by an infinite half-plane of inhomogeneous material. The method we pursue is quite different from that given by Luneberg.[7]

In subsequent papers, we shall discuss the application of these ideas to general hyperbolic systems, to partial differential equations of parabolic type, and to general operator equations of the form

$$u_{tt} + A^2u = 0, \tag{1.3}$$

where A is a positive definite operator. Furthermore, we shall consider spherical and cylindrical geometries.

2. *A Localization Device for Wave Processes.*—Consider a plane wave, $e^{i(k_0x-\omega t)}$ arriving from the homogeneous space $x < 0$ and approaching the inhomogeneous space $x > 0$. At the boundary, $x = 0$, the wave is split into a reflected wave and a refracted wave. Let us now suppose that there is an immediate reflected wave and refracted wave obtained by supposing that the inhomogeneous medium is actually homogeneous with wave number $k(+0)$. If the inhomogeneous region is now taken to be the limit of a sequence of interfaces, with this type of reflection and refraction occurring at each interface, we can obtain the total reflected wave and the disturbance inside the region $x > 0$ by adding up the effects of the reflected and refracted waves obtained in this way.

It is this principle which we wish to verify under appropriate assumptions for equations (1.1) and (1.2).

3. *The Bremmer Series.*—Let us define the sequence $\{u_n(x)\}$ where

$$\begin{aligned}
u_0(x) &= \left(\frac{k_0}{k(x)}\right)^{1/2} e^{i\int_0^x k(s)ds}, \\
u_1(x) &= \frac{-1}{2(k(x))^{1/2}} \int_x^\infty \frac{k'(s)}{k(s)^{1/2}}\, u_0(s) e^{i\int_x^s k(t)dt}\, ds, \\
u_{2N}(x) &= \frac{1}{2(k(x))^{1/2}} \int_0^x \frac{k'(s)}{k(s)^{1/2}} u_{2N-1}(s)\, e^{i\int_s^x k(t)dt}\, ds, \\
u_{2N+1}(x) &= -\frac{1}{2(k(x))^{1/2}} \int_x^\infty \frac{k'(s)}{k(s)^{1/2}}\, u_{2N}(s) e^{i\int_x^s k(t)dt}\, ds.
\end{aligned} \tag{3.1}$$

This series was derived by Bremmer,[5] using the localization principle of Section 2. Standard techniques yield

THEOREM 1. *If* $|k(x)| \geq a^2 > 0$, *for* $x \geq 0$, *and* $\int_0^\infty |k'(x)|dx < \infty$ *and is sufficiently small, then the Bremmer series,* $u(x) = \sum_{n=0}^{\infty} u_n(x)$, *converges and represents a solution of (1.1). Another linearly independent solution may be obtained by replacing* i *by* $-i$.

4. *The WKB Approximation for Matrix Systems.*—In order to obtain an analogue of this result for matrix systems of the form of (1.2), we introduce a matrix wave-function e^{iK_0x} (we shall omit the scalar term $e^{-i\omega t}$) associated with the homogeneous space $x < 0$ possessing the wave matrix K_0. Here e^{iK_0x} denotes the matrix exponential function.[6] At the interface, $x = 0$, between $x < 0$ and the inhomogeneous space $x > 0$, specified by e^{iK_1x}, there is a reflection and a refraction. The disturbance in $x < 0$ is given by $e^{iK_0x} + e^{-iK_0x}A$, the original wave plus a reflected wave, and there is a refracted wave in $x > 0$ given by $e^{iK_1x}B$, where the matrices A and B are determined by the continuity of the functions and their derivatives at $x = 0$. Hence

$$A = (K_1 + K_0)^{-1}(K_0 - K_1), \qquad B = 2(K_1 + K_0)^{-1}K_0. \tag{4.1}$$

Taking account only of the refractions, we obtain

THEOREM 2. *The WKB approximation to (1.2) is the solution of*

$$\frac{dV}{dx} = (iK(x) - {}^1/_2K^{-1}(x)K'(x))V(x), \qquad V(0) = I. \tag{4.2}$$

Under the assumption that $K(x)$ is positive definite for $x \geq 0$ and that $\int_0^\infty \|K'(x)\|dx$ is sufficiently small, it can be shown that $V(x)$ is the first term of a generalized Bremmer series which converges to a solution of (1.2).

5. *Reflection from an Inhomogeneous Space.*—Let us consider once again the scalar plane wave case. We wish to determine the reflected wave in $x < 0$ due to the inhomogeneous space $x > 0$. To obtain this, we consider the more general problem of determining the reflected wave from $x \geq z$ due to an incident wave $e^{ik_0(x-z)}$, where $k_0 = k(z - 0)$. Let the coefficient of $e^{-ik_0(x-z)}$ in $x < z$ be $u(z)$. Then, applying the localization principle of Section 2, we see that

$$u(z) = \frac{k(z) - k(z+\Delta)}{k(z) + k(z+\Delta)} + \frac{2k(z)}{k(z) + k(z+\Delta)}\, u(z+\Delta)\, \frac{2k(z+\Delta)}{k(z) + k(z+\Delta)} + \frac{2k(z)}{k(z) + k(z+\Delta)}\, u(z+\Delta)\, \frac{k(z+\Delta) - k(z)}{k(z) + k(z+\Delta)}\, u(z+\Delta) + o(\Delta), \quad (5.1)$$

which leads to the Riccati differential equation,

$$u'(z) = -\frac{k'}{k} + \frac{k'}{k}\, u^2. \quad (5.2)$$

If the medium is homogeneous for $z_0 < x$, with $k(x) = k_0$, then u must satisfy the boundary condition,

$$u(z_0) = \frac{k(z_0 - 0) - k_0}{k(z_0 - 0) + k_0}; \quad (5.3)$$

otherwise we employ the condition $\lim_{x \to \infty} u(z) = 0$.

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*INVARIANT IMBEDDING RELATION FOR THE PRINCIPLES OF INVARIANCE**

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1. *Introduction.*—The *principle of invariant imbedding*, recently stated by Bellman and Kalaba,[1] is a rule of action which may be followed in the mathematical formulation of any of a wide class of problems concerned with transfer phenomena in general media. The statement of the principle generalizes the methodology originally developed by Ambarzumian[2, 3] and extended by Chandrasekhar[4, 5] in their studies of the transfer of radiation through scattering and absorbing media. In this note we exhibit an explicit analytic embodiment of the principle for radiative-transfer and neutron-transport processes. This symbolic statement of the principle—which we shall call the *invariant imbedding relation*—yields in particular the general symmetric forms of the principles of invariance[5] for these processes. The semigroup features which are generally associated with an invariantly imbedded process are also implicit in the invariant imbedding relation.

The present discussion will be limited to the steady-state case on a class of inhomogeneous one-parameter carrier spaces[6] (e.g., plane-parallel, cylindrical, spherical, and general one-parameter space-filling families of surfaces). Additional features of the invariant imbedding relation such as those associated with time-dependence, polarization, and more general carrier spaces, will be reserved for subsequent discussions.

2. *Invariant Imbedding Relation.*—*Let* $\Phi = X \times \Xi$ *be a one-parameter carrier space in* E_3, *i.e.*, $X = \{X_x\colon \ x \in [a, b]\}$, *where* $[a, b]$ *is a closed interval in the extended real-number system, and* Ξ *is the unit sphere in* E_3. *For each* $y \in [a, b]$ *there is a pair* $[N_+(y), N_-(y)]$ *of (real or vector-valued) functions on* $\Phi_y = X_y \times \Xi_+$. *Let* $\mathfrak{N}$ *denote the collection of all ordered pairs of functions* $[N_+(z), N_-(x)]$, $[x, z] \subset [a, b]$, *with subcollections* $\mathfrak{N}_+$ *and* $\mathfrak{N}_-$ *defined as* $\{N_+(z);\ z \in [a, b]\}$ *and* $\{N_-(x)\colon x \in [a, b]\}$, *respectively. Then for each* $y \in [x, z] \subset [a, b]$, *there is a map* $\mathfrak{M}(x, y, z)$ *of* $\mathfrak{N}$ *into* $\mathfrak{N}$ *such that*

$$[N_+(y), N_-(y)] = [N_+(z), N_-(x)]\mathfrak{M}(x, y, z), \tag{1}$$

where

$$\mathfrak{M}(x, y, z) = \begin{pmatrix} \mathfrak{I}(z, y, x), & \mathfrak{R}(z, y, x) \\ \mathfrak{R}(x, y, z), & \mathfrak{I}(x, y, z) \end{pmatrix},$$

in which $\mathfrak{R}(x, y, z)$ *is the complete reflectance operator with domains* $\mathfrak{N}_+$, $\mathfrak{N}_-$, *and ranges* $\mathfrak{N}_-$, $\mathfrak{N}_+$, *respectively;* $\mathfrak{I}(x, y, z)$ *is the complete transmittance operator with domains* $\mathfrak{N}_+$, $\mathfrak{N}_-$, *and ranges* $\mathfrak{N}_+$, $\mathfrak{N}_-$, *respectively. In addition,* $\mathfrak{I}(x, z, z) = \mathrm{T}(x, z)$ *is the standard transmittance operator and* $\mathfrak{I}(x, x, z) = I$, *the identity operator;* $\mathfrak{R}(x, x, z) = \mathrm{R}(x, z)$ *is the standard reflectance operator and* $\mathfrak{R}(x, z, z) = O$, *the zero operator.*

Expression (1) is the *invariant imbedding relation* for the radiative-transfer and neutron-transport contexts. The pair of functions $N(y) = [N_+(y), N_-(y)]$ is the abstract version of the classical intensity (or radiance) function on a surface X_y, y units of depth "below" the upper boundary of the medium. $N_+(y)$ is a *function*

which physically describes the "outward"-directed flux and $N_-(y)$ describes the "inward"-directed flux at each point of X_y (Fig. 1). $N(y)$ need not be constant over X_y. Starting with the S and T functions of Chandrasekhar, one may obtain concrete examples of the various operators and of relation (1) in the classical case of a finitely deep plane-parallel medium.

3. *Principle of Invariant Imbedding.*—In the present context, the *invariant process* mentioned in the general statement of the principle is represented by the collection $\{\mathfrak{M}(x, y, z): y \in [x, z] \subset [a, b]\}$ of transformations, and the *state vectors* are the elements of the collection $\mathfrak{N}$.

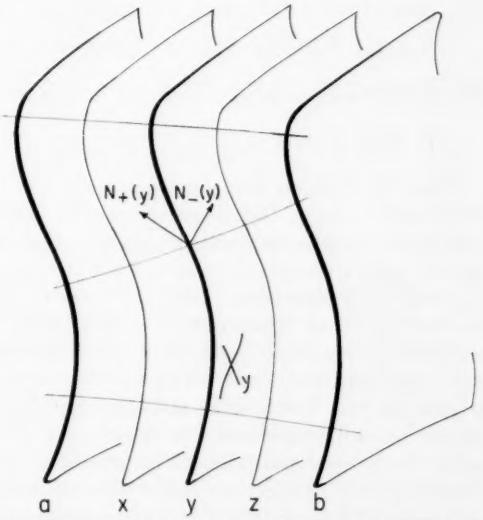

FIG. 1.—$N_+(y)$ and $N_-(y)$ describe the outward- and inward-directed flux across the surface x_y, respectively. Ξ_+, not explicitly shown, is the "outward" hemisphere of the unit sphere Ξ.

4. *Semigroup Properties of the Imbedding Relation.*—The semigroup relations are obtained by setting $N_+(b) = 0$ (the zero function on $X_b \times \Xi_+$). By equation (1), for all $y \in [a, b]$,

$$[N_+(y), N_-(y)] = [N_+(b), N_-(a)]\mathfrak{M}(a, y, b) = [N_-(a)\mathfrak{R}(a, y, b), N_-(a)\mathfrak{I}(a, y, b)],$$

and for $b \in [y, b]$,

$$[N_+(b), N_-(b)] = [N_+(b), N_-(y)]\mathfrak{M}(y, b, b) = [0, N_-(y)\mathfrak{I}(y, b, b)],$$

whence

$$N_-(b) = N_-(a)\mathfrak{I}(a, b, b) = N_-(a)\mathfrak{I}(a, y, b)\mathfrak{I}(y, b, b),$$

so that

$$\mathfrak{I}(a, b, b) = \mathfrak{I}(a, y, b)\mathfrak{I}(y, b, b), \qquad y \in [a, b]. \tag{2}$$

In a similar way it may be shown that

$$\mathfrak{R}(a, z, b) = \mathfrak{I}(a, y, b)\mathfrak{R}(y, z, b), \qquad y \in [a, z]. \tag{3}$$

The complementary set of semigroup relations are obtained by setting $N_-(a) = 0$.

5. *Principles of Invariance.*—The four general principles of invariance associated with X are obtained by first writing out in full the matrical relation (1) and then suitably choosing the spread of the interval $[x, z]$ and the location of the parameter y in $[x, z]$. Thus, from (1):

I. Let $x = y$, use component $N_+(y)$:

$$N_+(y) = N_+(z)T(z, y) + N_-(y)R(y, z).$$

II. Let $z = y$, use component $N_-(y)$:

$$N_-(y) = N_-(x)T(x, y) + N_+(y)R(y, x).$$

III. Use I twice: Let $y = a$, $z = b$; then $y = a$, z arbitrary:

$$N_+(a) = N_+(b)\mathrm{T}(b, a) + N_-(a)R(a, b) = N_+(z)T(z, a) + N_-(a)R(a, z).$$

IV. Use II twice: Let $y = b$, $x = a$; then $y = b$, x arbitrary:

$$N_-(b) = N_-(a)T(a, b) + N_+(b)R(b, a) = N_-(x)T(x, b) + N_+(b)R(b, x).$$

Three observations may be made here: (1) the four principles of invariance stated above are in *undecomposed* operator form. The advantage gained by retaining this form is the resultant general symmetry of the statements. (2) Principles III and IV are special cases of I and II, respectively, and the latter in turn are obtained by the appropriate permutation of $+$, $-$, and x, z, in a single basic statement derived from expression (1). (3) The invariant imbedding relation and the principles of invariance refer (as do their classical counterparts) to an arbitrary source-free subset of X defined by the interval $[x, z]$. This is in keeping with the original purpose[2-5] of defining and characterizing the inherent reflecting and transmitting properties of source-free subsets of a space. Thus the problem of formulating the general principles of invariance is, by definition, independent of the boundary-value problem associated with *internally* distributed sources. It should be observed, however, that if sources are present *in* X, relation (1) and its corollaries I–IV continue to hold for all those subsets (defined by $[x, z]$) which are free of sources.

The classical forms of the principles of invariance are obtained by: (1) decomposing the functions $N(y)$, $y \in [a, b]$ into the sum of their reduced and diffuse components: $N(y) = N^\circ(y) + N^*(y)$; (2) imposing the boundary conditions: $N_-(a) = N^\circ(a)$ with Dirac-delta structure over Ξ_+, and $N_+(b) = 0$; (3) assuming X to be a plane-parallel homogeneous slab, so that $R(x, y) = R(y, x) = R(|x - y|)$ and $T(x, y) = T(y, x) = T(|x - y|)$.

The decomposition of $N(y)$ carries through to the general case, and the classical boundary conditions may be imposed on $N_-(a)$ and $N_+(b)$. However, the general inhomogeneity and curvilinear structure of X, first of all, give rise to the *polarity* of the R and T operators: $R(x, y) \neq R(y, x)$, $T(x, y) \neq T(y, x)$; and, second, remove some of the original invariant features of the R and T operators such as those associated with the physical operations of adding or subtracting finite layers of the media. The general functional relations satisfied by the R and T operators will be considered at a later time.

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*FUNCTIONAL RELATIONS FOR THE R AND T OPERATORS ON PLANE-PARALLEL MEDIA**

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1. *Introduction.*—In the classical theory of radiative transfer, one may associate with a separable plane-parallel medium—i.e., a medium in which the phase function $p = 4\pi\sigma/\alpha$ is independent of depth—two functions, S and T, which analytically characterize the diffuse reflectance and transmittance of the medium. Explicit knowledge of the S and T functions is tantamount to a solution of an important class of multiple-scattering problems associated with the medium. Whereas the volume-scattering function, σ, and the volume-attenuation function, α, summarize the *local* optical properties of the medium, the S and T functions summarize the *global* optical properties of the medium. Furthermore, as the local behavior of the light field is described by the equation of transfer in terms of σ and α, so is the global behavior of the light field described by the principles of invariance in terms of S and T. The global approach was initiated by Ambarzumian, but it remained for Chandrasekhar to formulate the four principles of invariance and deduce from them the requisite integral equations for S and T for the case of finite, separable plane-parallel media. The importance of the global formulation has been illustrated by its role in many successful solutions of multiple-scattering problems which were intractable under the local formulation.

In this note we consider the case of finite, *non-separable* plane-parallel media. As is shown below, the study of these media requires not two but four functions: a reflectance-transmittance pair for each of the two boundaries of the medium. The four principles of invariance for a non-separable plane-parallel medium are formulated and are found to be just sufficient to yield the appropriate integrodifferential equations governing the four functions.

It is of some interest to observe that the following derivations can be patterned after the procedure used by Chandrasekhar for the separable case, namely, the procedure which makes explicit use of the principles of invariance in conjunction with the equation of transfer. This procedure promises to be adequate, under appropriate extensions, for use in the formulation of the functional relations governing the scattering functions in general one-parameter carrier spaces. In this way a useful methodology already extant in classical theory is retained and is extended to

more general settings. In addition, this procedure appears to be pertinent to an important question in the mathematical foundations of radiative-transfer theory, namely, the question of the apparent mathematical abiogenesis of the S and T functions and the principles of invariance. In short, are the principles of invariance part of the basic laws of radiative-transfer theory or merely consequences of them? In this connection, we observe that the operator form of the equation of transfer adopted during the course of the present derivations yields a pair of statements which clearly represent what might be termed the *local* forms of the principles of invariance. These in turn may possibly supply a key, by means of the general theory of Green's functions for linear operators, to an existence proof for the S and T functions and perhaps a *derivation* of the statements of the principles of invariance themselves. If this or any coterminous procedure is possible, then the general equation of transfer may serve as the sole starting point of an unbroken network of deductions covering all salient mathematical features of modern radiative-transfer theory.

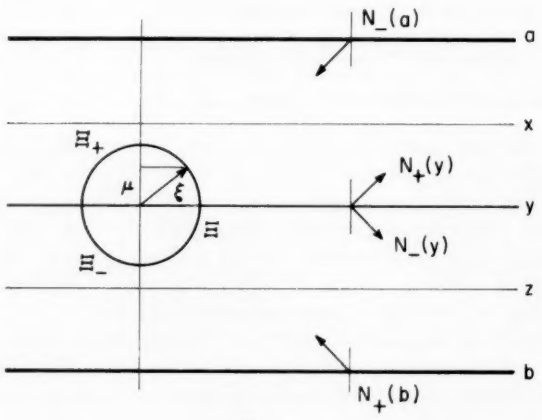

FIG. 1

The operators used in the present note are left in undecomposed form in order to secure maximum symmetry and compactness of the formulations.

2. *Equation of Transfer and the Local Forms of the Principles of Invariance.*— We begin with a plane-parallel medium imbedded in Euclidean three space (Fig. 1), namely, that subset contained by two planes at geometric depths a and b from some datum plane. Several intermediate planes at depths x, y, z will also be considered: $a \leq x \leq y \leq z \leq b$. The volume-attenuation and volume-scattering functions, α and σ, along with the radiance function (specific intensity) N, will depend spatially only on the depth parameters and in an arbitrary way. The steady-state, emission-free equation of transfer for radiance may be written

$$\mu \frac{dN(y, \mu, \phi)}{dy} = -\alpha(y)N(y, \mu, \phi) + N_*(y, \mu, \phi), \tag{1}$$

where

$$N_*(y, \mu, \phi) = \int_{\Xi} \sigma(y; \mu, \phi; \mu', \phi')N(y, \mu', \phi')d\mu'd\phi'.$$

We adopt the concepts of outward and inward radiances: $N(y, +\mu, \phi)$, $N(y, -\mu, \phi)$, $0 < \mu \leq 1$, and write the outward and inward radiance distributions at level y as

$$N_+(y), \qquad N_-(y);$$

these are *functions* which assign to the pair (μ, ϕ), $0 < \mu \leq 1, 0 < \phi \leq 2\pi$ the appropriate radiance values at level y in the indicated directions. If Ξ_+ and Ξ_- are the totalities of outward and inward unit vectors at each level y, the equation of transfer may be written in the following operator form:

$$-\frac{dN_+(y)}{dy} = N_+(y)\tau(y) + N_-(y)\rho(y),$$

$$\frac{dN_-(y)}{dy} = N_-(y)\tau(y) + N_+(y)\rho(y), \tag{2}$$

where, for each y, $a \leq y \leq b$, and arbitrary (μ, ϕ), $0 < \mu \leq 1, 0 \leq \phi < 2\pi$,

$$\rho(y) = \frac{1}{\mu}\int_{\Xi_+} \sigma_-(y; \mu, \phi; \mu', \phi')[\quad]d\mu' d\phi',$$

$$\tau(y) = \frac{1}{\mu}\int_{\Xi_+} \sigma_+(y; \mu, \phi; \mu', \phi')[\quad]d\mu' d\phi' - \frac{1}{\mu}\alpha(y).$$

Thus $\rho(y)$ and $\tau(y)$ evidently play the role of local reflectance and transmittance operators, and σ_- and σ_+ are appropriate restrictions of σ chosen so that their μ-arguments are positive. (Such restrictions are possible because of the implicitly assumed isotropy of the medium.) The pair (2) clearly represents the *local* (or integrodifferential) forms of the principles of invariance.

3. *Principles of Invariance for Non-separable Plane-Parallel Media.*—In an earlier note[1] the classical principles of invariance were generalized to the context of general one-parameter carrier spaces. These generalizations were based directly on considerations of the classical forms as given by Chandrasekhar and resulted in an explicit analytical embodiment of the principle of invariant imbedding[2] for the radiative-transfer context. The two main statements of the principles of invariance were shown to be of the forms:

$$\left.\begin{array}{ll} \text{I.} & N_+(y) = N_+(z)T(z, y) + N_-(y)R(y, z) \\ \text{II.} & N_-(y) = N_-(x)T(x, y) + N_+(y)R(y, x) \end{array}\right\} a \leq x \leq y \leq z \leq b. \tag{3}$$

The structural similarity of sets (2) and (3) can be noted. By suitably choosing the spread of the interval $[x, z]$ and the location of y within it, the operator forms of four principles of invariance for the present context may be obtained from (3):

$$\begin{array}{lll} \text{I.} & N_+(y) = N_+(b)T(b, y) + N_-(y)R(y, b), & \\ \text{II.} & N_-(y) = N_-(a)T(a, y) + N_+(y)R(y, a), & \\ \text{III.} & N_+(a) = N_+(b)T(b, a) + N_-(a)R(a, b) = N_+(y)T(y, a) + N_-(a)R(a, y), & \\ \text{IV.} & N_-(b) = N_-(a)T(a, b) + N_+(b)R(b, a) = N_-(y)T(y, b) + N_+(b)R(b, y). & \end{array}$$

For every pair of depths (x, z) in the closed interval $[a, b]$ we have

$$R(x, z) = \frac{1}{\mu}\int_{\Xi_+} R(x, z; \mu, \phi; \mu', \phi')[\quad]d\mu' d\phi'.$$

Furthermore,

$$T(x, z) = T^\circ(x, z) + T^*(x, z),$$

where

$$T^*(x, z) = \frac{1}{\mu}\int_{\Xi_+} T(x, z; \mu, \phi; \mu', \phi')[\quad]d\mu' d\phi',$$

$$T^\circ(x, z) = \int_{\Xi_+} T_r(x)\delta(\mu - \mu')\delta(\phi - \phi')[\quad]d\mu' d\phi',$$

in which

$$T_r(x) = \exp\left\{-\frac{1}{\mu'}\left|\int_x^z \alpha(y)dy\right|\right\}, \qquad r = |z - x|/\mu', \qquad 0 < \mu' \leq 1.$$

$T(x, z)$ is the *transmittance operator:* $T^\circ(x, z)$, $T^*(x, z)$ are its *reduced* and *diffuse* components. $R(x, z)$ is the *reflectance operator*, and, since the reflected flux is, by definition, entirely of diffuse character, the corresponding reduced component $R^\circ(x, z)$ of $R(x, z)$ is the zero operator, so that $R^*(x, z) = R(x, z)$. It may be shown that, in analogy to the S and T functions of the classical theory, the present R and T functions have the properties:

$$\lim_{z \to x} R(x, z; \mu, \phi; \mu', \phi')/|z - x| = \sigma_-(x; \mu, \phi; \mu', \phi'),$$
$$\lim_{z \to x} T(x, z; \mu, \phi; \mu', \phi')/|z - x| = \sigma_+(x; \mu, \phi; \mu', \phi').$$

4. *Functional Relations for the R and T Operators.*—In what follows, let $N_-(a)$ be of arbitrary angular structure and set $N_+(b) = 0$ (the zero function); furthermore, all functions and operators will be assumed continuously differentiable with respect to the depth parameter, y. Now from I, with the adopted boundary conditions in force,

$$\frac{dN_+(y)}{dy} = \frac{dN_-(y)}{dy}R(y, b) + N_-(y)\frac{dR(y, b)}{dy}. \tag{4}$$

Since interest is at present centered on the functional relations for the slab defined by the planes at depths a and b, we consider

$$\lim_{y \to a} \frac{dN_+(y)}{dy},$$

which, by (2), has the form

$$-[N_+(a)\tau(a) + N_-(a)\rho(a)] = -N_-(a)[R(a, b)\tau(a) + \rho(a)],$$

where the latter relation follows from use of the left-hand equality of III. Similarly,

$$\lim_{y \to a} \frac{dN_-(y)}{dy} = N_-(a)[\tau(a) + R(a, b)\rho(a)].$$

Since

$$\lim_{y \to a} \frac{dR(y, b)}{dy} = \frac{\partial R(a, b)}{\partial a},$$

and $N_-(a)$ is arbitrary, the limit operation $\lim_{y \to a}$ applied to (4) yields

$$\text{I}'. \quad -\frac{\partial R(a, b)}{\partial a} = \rho(a) + \tau(a)R(a, b) + R(a, b)\tau(a) + R(a, b)\rho(a)R(a, b).$$

Now, from II,

$$\frac{dN_-(y)}{dy} = N_-(a)\frac{dT(a, y)}{dy} + \frac{dN_+(y)}{dy}R(y, a) + N_+(y)\frac{dR(y, a)}{dy}. \tag{5}$$

By (2) and the left-hand equality of IV,

$$\lim_{y \to b} \frac{dN_-(y)}{dy} = N_-(a)T(a, b)\tau(b),$$

$$\lim_{y \to b} \frac{dN_+(y)}{dy} = -N_-(a)T(a, b)\rho(b).$$

Applying the limit operation $\lim_{y \to b}$ to (5), setting

$$\frac{\partial T(a, b)}{\partial b} = \lim_{y \to b} \frac{dT(a, y)}{dy},$$

and recalling that $N_-(a)$ is arbitrary, we have

$$\text{II}'. \quad \frac{\partial T(a, b)}{\partial b} = T(a, b)\tau(b) + T(a, b)\rho(b)R(b, a).$$

The procedure is now clear. By applying this procedure in turn to the right-hand equalities of principles III and IV, we have the results:

$$\text{III}'. \quad \frac{\partial R(a, b)}{\partial b} = T(a, b)\rho(b)T(b, a),$$

$$\text{IV}'. \quad -\frac{\partial T(a, b)}{\partial a} = \tau(a)T(a, b) + R(a, b)\rho(a)T(a, b).$$

In conclusion, several general observations may be made. (1) For non-separable spaces, i.e., spaces in which the phase function $p = 4\pi\sigma/\alpha$ is dependent on depth, I′–IV′ show that the R and T operators possess *polarity:* $R(a, b) \neq R(b, a)$; $T(a, b) \neq T(b, a)$. (2) The system I′–IV′ is a simultaneous system of the simplest kind. It can be solved, in principle, by successively considering the individual relations in either of the following orders: I′, IV′, III′, II′; I′, IV′, II′, III′. (3) To obtain the equations governing the R and T *functions*, one must first assume, as in the classical case, a Dirac-delta structure for $N_-(a)$: $N_-(a) = N^\circ\delta(\mu - \mu')\delta(\phi - \phi')$. Then, by successively applying the operators on each side of the statements I′–IV′ to $N_-(a)$ and making the appropriate reductions, the requisite integrodifferential equations for the R and T functions are obtained.

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*TIME-DEPENDENT PRINCIPLES OF INVARIANCE**

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1. *Introduction.*—Radiative-transfer phenomena, whether in the transient or stationary states, may be classified according to the relative importance of the roles played by the three general processes of conduction, convection, and radiation. *Strict radiative transfer* refers to the transfer by radiation processes alone. In 1926 work on time-dependent strict radiative-transfer processes had been carried out by Milne and later extended by Chandrasekhar.[1] In these studies the time-dependent radiation field is considered to be governed principally by the various decay rates of excited atoms. This is in contrast to the time-dependent process initiated and sustained by the mechanism of simple scattering and the speed of propagation of radiant energy through the medium (the multiple-scattering process). In another early study, simultaneous consideration of time-dependent convection and radiation processes was made by Rosseland.[2] Aside from these studies, the time-dependent case of strict radiative-transfer theory has been relatively unexplored. This is particularly true for the time-dependent multiple-scattering problem.

Most of the tools presently available for the study of time-dependent multiple-scattering phenomena are imports from neutron-transport theory. In this field the approach to the transient case has been successfully effected by means of the linearized Boltzmann transport equation—the exact counterpart to the equation of transfer in radiative-transfer theory. The approach may be analyzed into two alternate procedures: the direct and the indirect. The direct procedure, as exemplified in the papers of Lehner and Wing,[3] is based on the transport equation, in which the time-dependence is explicitly retained throughout. In the indirect procedure, the time-dependent problem is immediately shunted to a stationary problem by means of the Laplace transform, is solved in that context, and then is switched back to a non-stationary problem by an inverse Laplace transform. Examples may be found in the works of Grosjean,[4] Case, de Hoffman, and Placzek,[5] and Davison.[6] We note that this procedure is possible only if the attenuation and scattering functions are time-independent. The mathematical questions of the existence of solutions sought in the time-dependent case by these methods can be settled, for example, by applications of the results obtained by Kurth.[7]

There remains, however, to explore a potentially useful approach to the time-dependent multiple-scattering processes—an approach based on the principle of invariance formulations which have been instrumental in the exact solution of a large class of stationary multiple-scattering problems in radiative-transfer theory. In this note we exhibit the formulation of the requisite statements of the four time-dependent principles of invariance and show that they are sufficient to derive the functional relations governing the time-dependent R and T functions and their corresponding integral operators. The derivations follow closely the pattern used in some earlier studies of the stationary case.[8] In this way we add to the evidence that the classical procedures initiated by Chandrasekhar can be extended to more general settings. For brevity, the present discussion is limited to plane-parallel

media. However, the media will be assumed finite, non-separable, and such that the attenuation and scattering functions are generally dependent on time.

2. *Local Forms of the Time-dependent Principles of Invariance.*—With the exception of the explicit incorporation of time parameters, the geometrical setting and the notation used in the present investigation are the same as those used in the study of the steady-state case.[8] In particular, the time-dependent equation of transfer will be written

$$\mu \frac{\partial N(y, t, \mu, \phi)}{\partial y} + \frac{1}{v} \frac{\partial N(y, t, \mu, \phi)}{\partial t} = -\alpha(y, t)N(y, t, \mu, \phi) + N_*(y, t, \mu, \phi) \quad (1)$$

where

$$N_*(y, t, \mu, \phi) = \int_\Xi \sigma(y, t; \mu, \phi; \mu', \phi')N(y, t, \mu', \phi')d\mu'd\phi'.$$

As before, we use the notions of outward and inward radiance distributions:

$$N_+(y, t), \qquad N_-(y, t).$$

The local reflectance and transmittance operators are defined as

$$\rho(y, t) = \frac{1}{\mu} \int_{\Xi_+} \sigma_-(y, t; \mu, \phi; \mu', \phi')[\quad]d\mu'd\phi',$$

$$\tau(y, t) = \frac{1}{\mu} \int_{\Xi_+} \sigma_+(y, t; \mu, \phi; \mu', \phi')[\quad]d\mu'd\phi' - \frac{1}{\mu} \alpha(y, t),$$

so that the local forms of the time-dependent principles of invariance may be written thus:

$$\frac{D_-N_+(y, t)}{Dy} = -\frac{\partial N_+(y, t)}{\partial y} + \frac{1}{\mu v} \frac{\partial N_+(y, t)}{\partial t} = N_+(y, t)\tau(y, t) + N_-(y, t)\rho(y, t), \quad (2)$$

$$\frac{D_+N_-(y, t)}{Dy} = \frac{\partial N_-(y, t)}{\partial y} + \frac{1}{\mu v} \frac{\partial N_-(y, t)}{\partial t} = N_-(y, t)\tau(y, t) + N_+(y, t)\rho(y, t).$$

3. *Time-dependent R and T Operators and Principles of Invariance.*—For every pair (x, z) of geometric depths and ordered pair (t', t) of times, $t' \leq t$, we define two non-negative real-valued functions $R(x, z, t', t; \ ; \ )$, $T(x, z; t'; t; \ ; \ )$, on $\Xi_+ \times \Xi_+$ and their associated integral operators:

$$R(x, z, t', t) = \frac{1}{\mu} \int_{\Xi_+} R(x, z, t', t; \mu, \phi; \mu', \phi')[\quad]d\mu'd\phi',$$

$$T^*(x, z, t', t) = \frac{1}{\mu} \int_{\Xi_+} T(x, z, t', t; \mu, \phi; \mu', \phi')[\quad]d\mu'd\phi'.$$

These are clearly analogous to the operators $R(x, z)$ and $T^*(x, z)$ considered in the steady-state case. In addition, we define

$$T^\circ(x, z, t', t) = \int_{\Xi_+} T_r(x)\delta(\mu - \mu')\delta(\phi - \phi')\delta\left(t - \frac{r}{v} - t'\right)[\quad]d\mu'd\phi',$$

where $T_r(x)$, $r = |z - x|/\mu'$, is defined as in the stationary case. Set

$$T(x, z, t', t) = T^{\circ}(x, z, t', t) + T^{*}(x, z, t', t).$$

In addition to the usual analytic conditions imposed on the R and T functions in the stationary context, we require at present three additional properties: (i) If $N_-(x, \cdot)$ is arbitrary and $N_+(z, \cdot) = 0$, then

$$N_+(x, t) = \int_{-\infty}^{t} N_-(x, t')R(x, z, t', t)dt' = N_-(x, \bar{t}')R(x, z, \bar{t}', t),$$

$$N_-(z, t) = \int_{-\infty}^{t - \frac{|z-x|}{v}} N_-(x, t')T(x, z, t', t)dt' = N_-(x, \bar{t}')T(x, z, \bar{t}', t).$$

The right-hand equalities define a convenient integration convention. (ii) In particular, if $N_-(x, \cdot)$ is constant over $(-\infty, t)$, t finite, and α and σ are independent of t, then

$$N_+(x) = N_-(x, \bar{t}')R(x, z, \bar{t}', t) = N_-(x)R(x, z),$$
$$N_-(z) = N_-(x, \bar{t}')T(x, z, \bar{t}', t) = N_-(x)T(x, z),$$

where $R(x, z)$ and $T(x, z)$ are the steady-state operators considered earlier. (iii) Finally,

$$R(x, z, t, t) = 0 \qquad \text{for all } t,$$

and

$$\mathrm{T}(x, z, t', t) = 0 \qquad \text{for} \qquad t - t' \leq |z - x|/v.$$

These adopted conditions are sufficient to allow the formulation of both the time-dependent principles of invariance and the necessary functional relations governing the time-dependent R and T operators which reduce, under steady-state conditions, to their respective steady-state counterparts.

The two main time-dependent principles of invariance may then be written:

$$\begin{aligned} &\text{I.} \quad N_+(y, t) = N_+(z, \bar{t}')T(z, y, \bar{t}', t) + N_-(y, \bar{t}')R(y, z, \bar{t}', t), \\ &\text{II.} \quad N_-(y, t) = N_-(x, \bar{t}')T(x, y, \bar{t}', t) + N_+(y, \bar{t}')R(y, x, \bar{t}', t), \\ &\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad a \leq x \leq y \leq z \leq b. \end{aligned} \tag{3}$$

The structural similarity of sets (2) and (3) can be noted. Set (3) yields, after appropriate choices of the spread of $[x, z]$ and the location of y within it, the operator forms of the four principles of invariance for the present context. The general resultant forms, being structured similarly to the steady-state set considered earlier, need not be given here. Instead, for brevity, we assume that the usual boundary conditions are already in force, namely, $N_-(a, \cdot)$ is arbitrary, and $N_+(b, \cdot) = 0$ (the zero function). The appropriate principles then follow directly from set (3):

$$\begin{aligned} &\text{I.} \quad N_+(y, t) = N_-(y, \bar{t}')R(y, b, \bar{t}', t), \\ &\text{II.} \quad N_-(y, t) = N_-(a, \bar{t}')T(a, y, \bar{t}', t) + N_+(y, \bar{t}')R(y, a, \bar{t}', t), \\ &\text{III.} \quad N_+(a, t) = N_-(a, \bar{t}')R(a, b, \bar{t}', t) = N_+(y, t')T(y, a, \bar{t}', t) \\ &\qquad\qquad\qquad\qquad\qquad\qquad + N_-(a, \bar{t}')R(a, y, \bar{t}', t), \\ &\text{IV.} \quad N_-(b, t) = N_-(a, \bar{t}')T(a, b, \bar{t}', t) = N_-(y, \bar{t}')T(y, b, \bar{t}', t). \end{aligned}$$

4. *Functional Relations for the Time-dependent R and T Operators.*—In what follows, let $N_-(a, \cdot)$ be of arbitrary angular structure, but of Dirac-delta time-dependence: $N_-(a, t) = N_-(a, t_0)\delta(t - t_0)$. This procedure results in relatively simple relations for the R and T *operators* [cf. with the adoption of a Dirac-delta angular structure for $N_-(a)$ in the steady-state formulation which results in the relatively simpler relations for the R and T *functions*]. The subsequent operations involving the Dirac-delta functions, their products, and derivatives are governed by the usual conventions.[9]

We begin by applying the operator D_-/Dy to I:

$$\frac{D_-N_+(y, t)}{Dy} = \frac{D_-N_-(y, \bar{l}')}{Dy} R(y, b, \bar{l}', t) + N_-(y, \bar{l}') \frac{D_-R(y, b, \bar{l}', t)}{Dy}. \tag{4}$$

To this we apply in turn the operation $\lim_{y \to a}$. The left side of equation (4) yields, by means of (2),

$$N_+(a, t)\tau(a, t) + N_-(a, t)\rho(a, t) = N_-(a, t_0)[R(a, b, t_0, t)\tau(a, t) + \delta(t - t_0)\rho(a, t)],$$

the alternate expression comes from the left-hand side of III and the adopted form of $N_-(a, \cdot)$. Further, by (2),

$$\frac{D_-N_-(y,t')}{Dy} = -\frac{\partial N_-(y, t')}{\partial y} = \frac{1}{\mu v}\frac{\partial N_-(y, t')}{\partial t} - N_-(y, t')\tau(y, t') - N_+(y, t')\rho(y, t'),$$

so that

$$\lim_{y \to a} \frac{D_-N_-(y, t')}{Dy} = N_-(a, t_0) \left[\frac{1}{\mu v}\delta'(t' - t_0) - \delta(t' - t_0)\tau(a, t') - R(a, b, t_0, t')\rho(a, t')\right],$$

where $\delta'(t' - t_0)$ is the symbolic derivative of the Dirac-delta function with respect to t'. Finally,

$$\lim_{y \to a} \frac{D_-R(y, b, t', t)}{Dy} = -\frac{\partial R(a, b, t', t)}{\partial a} + \frac{1}{\mu v}\frac{\partial R(a, b, t', t)}{\partial t}.$$

Using these results and the fact that $N_-(a, \cdot)$ is of arbitrary angular structure, we have

$$\text{I}'. \quad -\frac{\partial R(a, b, t_0, t)}{\partial a} + \frac{1}{\mu v}\left[-\frac{\partial R(a, b, t_0, t)}{\partial t_0} + \frac{\partial R(a, b, t_0, t)}{\partial t}\right] = \delta(t - t_0)\rho(a,t) + \tau(a, t_0)R(a, b, t_0, t) + R(a, b, t_0, t)\tau(a, t) + R(a, b, t_0, \bar{l}')\rho(a, \bar{l}')R(a, b, \bar{l}', t).$$

Now starting with II, applying in turn the operations D_+/Dy and $\lim_{y \to b}$ and then making use of (2) and the left side of IV, we have, in a similar way,

$$\text{II}'. \quad \frac{\partial T(a, b, t_0, t)}{\partial b} + \frac{1}{\mu v}\frac{\partial T(a, b, t_0, t)}{\partial t} = T(a, b, t_0, t)\tau(b, t) + T(a, b, t_0, \bar{l}')\rho(b, \bar{l}')R(b, a, \bar{l}', t).$$

The third relation follows from the right-hand equality of III by applying the procedure used for I′. The result is

$$\text{III}'. \qquad \frac{\partial R(a, b, t_0, t)}{\partial b} = T(a, b, t_0, \bar{l}')\rho(b, \bar{l}')T(b, a, \bar{l}', t).$$

The fourth relation follows from the right-hand equality of IV by applying the procedure used for II′. The result is

$$\text{IV}'. \qquad -\frac{\partial T(a, b, t_0, t)}{\partial a} - \frac{1}{\mu v}\frac{\partial T(a, b, t_0, t)}{\partial l_0} = \tau(a, t_0)T(a, b, t_0, t) + R(a, b, t_0, \bar{l}')\rho(a, \bar{l}')T(a, b, \bar{l}', t).$$

We conclude by observing that (1) the time-dependent R and T operators will be homogeneous with respect to time (i.e., will depend on time differences $t - t'$) if and only if the attenuation and scattering functions are independent of t; (2) furthermore, for non-separable spaces, the time-dependent R and T operators exhibit polarity: $R(a, b, t', t) \neq R(b, a, t', t)$, $T(a, b, t', t) \neq T(b, a, t', t)$; (3) the solution procedures for the time-dependent relations are, in principle, the same as those followed in the stationary case.

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*ABERRANT SEGREGATION AND PSEUDOALLELISM AT THE GARNET LOCUS IN DROSOPHILA MELANOGASTER**

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The now common phenomenon of pseudoallelism has served to focus attention upon the operational nature of our notions concerning the structural and functional organization of the genetic material.[1, 2] Investigations of the spatial relationships of functionally related alterations of the genetic material in *Drosophila* consistently reveal a small number of distinct genetic loci. Moreover, clustered about each of the spatially distinct loci, there are mutants which have not exhibited recombination *inter se;* and these constitute members of a multiple allelic system in the classic sense.[3] These observations suggest a genetic fine structure not sensibly altered from the classic view of discrete units separated by non-genic materials, with recombination restricted to the non-genic, interstitial, regions. By way of contrast, similar systems in micro-organisms[2, 4] exhibit a high degree of spatial resolution. Such systems lend favor to the notion of a segment of genetic material, to be effectively a continuum, capable of alteration at many separable sites.

Recently, several cases of aberrant segregations, termed "gene conversions," have been found in microbial studies.[5] These cases parallel the behavior of the "mutable" reddish-alpha mutant of *Drosophila virilis.*[6]

This report provides evidence for pseudoallelism at the garnet locus in *D. melanogaster.* Evidence is presented which supports the view that the apparent difference between the *Drosophila* and microbial studies does not reflect a fundamental difference in the structural organization of the genetic material in these forms. Rather, it is suggested that the discrepancy between the *Drosophila* and microbial studies reflects observations made on similar structures at differing levels of resolution.

In addition to pseudoallelism at the garnet locus, there are recovered instances of aberrant segregations. Both the pseudoallelic recombinants and aberrant segregants together resemble markedly the behavior of reddish-alpha mutant of *D. virilis.*[6]

The garnet mutants of *D. melanogaster* are located in the vicinity of 44.4 on the X chromosome. These mutants alter the pigmentation of the eyes and larval Malphigian tubules. In this study, attention is restricted to mutants which are clearly distinct from wild type. All the mutants are recessive to wild type, and all heterozygotes between the garnet mutants exhibit a garnet phenotype.

Females were produced which were heterozygous for various combinations of garnet mutants and also heterozygous for markers adjacent to garnet. Several heterozygous combinations of autosomal inversions were tested in an attempt to increase recombination frequency in the garnet region of the X chromosome. No significant increases were found with any combination of inversions, and no further reference will be made to the autosome chromosome constitutions. Assay of segregating X chromosomes in heterozygotes was made by crossing to males with X chromosomes carrying a garnet mutant and one of the closely linked markers.

In Table 1, there are recorded data collected in six categories of experiments in-

volving an assay of over 750,000 segregating X chromosomes from heterozygous females. Most of the assayed chromosomes carried a garnet mutant and may, or may not, have carried one or more of the closely linked markers.

TABLE 1

ASSAY OF SEGREGATING X-CHROMOSOMES IN FEMALES OF THE INDICATED GENOTYPES

GROUP	Female Genotype	Non-Garnet Chromosomes Recovered*					Total Chromosomes Assayed	Per Cent Recombination
		s + pl	s + +	+ + +	dy + pl	dy + +		
1 A...	s g +/+ g^2 pl	2	0	0	...	...	68,051	
1 B...	dy g +/+ g^2 pl	...	...	0	1	0	65,251	0.0045
2	s g^3 +/+ g^2 pl	4	1*	1*	...	...	181,102	0.0044
3	s g^{50e} +/+ g^2 pl	2	1*	0	...	...	73,499	0.0054
4	s g^{50e} +/+ g^3 pl	...	1*	0	...	...	22,857	...
5	dy g pl/+ g^{50e} +	...	...	2*	...	2	165,182	0.0024
6	dy g pl/+ g^3 +	...	...	0	0	0	186,487	...
Total		8	3	3	1	2	762,429	...

* dy = dusky; s = sable; g = garnet; pl = pleated.

dy	s	g	pl
36.2	43.0	44.4	47.9

Nineteen exceptional (non-garnet) individuals were recovered. These were subjected to further breeding experiments designed to verify their X chromosomal constitutions. Two of these did not reproduce and are omitted from the tabulation. Verification of X-chromosomal constitution is particularly important in this study, since one of the markers (pleated) overlaps wild type but is recognizable in 70 per cent of the offspring possessing it. Moreover, such verification is of import because of the aberrant segregants recovered in this study. The exceptional non-garnet males which arose in this investigation were crossed to attached-X females. Their X-chromosomal constitutions were then determined by examination of male offspring. The exceptional non-garnet females were crossed to wild type (Oregon-R) males, and the genetic constitution of the X-chromosomes of these females were assayed by examination of male offspring.

The seventeen exceptional offspring which reproduced fell into two classes with respect to their X-chromosomal constitutions. Eleven individuals possessed g^+-bearing X chromosomes associated with recombination between the closely linked markers in a regular fashion (non-starred chromosomes, Table 1). The recovery of such non-garnet chromosomes associated with a specific pattern of marker recombination indicates that the garnet mutants are spatially separable, and the following linkage relations are indicated: The recombinations of Groups 1, 2, and 3 (Table 1) indicate that g^2 lies to the left of g, g^3, and g^{50e}. The Group 5 recombinations establish g^{50e} to the left of g, and the following map may be drawn:

dy. . . . s. . . . g^2. . . . g^{50e}. . . . g. . . .pl

The inability to recover recombinations between g^3 and g^{50e} or g places g^3 in the right end of the garnet segment, very close to g. It should be noted that double mutant chromosomes have not been recovered in these studies. The inability to recover the reciprocal double mutants is taken to mean that they are indistinguishable from single mutants by visual observation. The recombination percentages of Table 1 were obtained by multiplying by 2 to account for the missing class of

double mutants. One discrepancy should be noted. The distance between g^2 and g^{50e} should be less than the distance between g^2 and g or g^3. The calculations of Table 1 indicate a larger value for the distance between g^2 and g^{50e}. However, this discrepancy lies within the limits of sampling variation.

In addition to the recovery of non-garnet chromosomes associated with marker recombination, there were recovered six non-garnet chromosomes not conforming to the pattern of regular marker recombination following crossover between pseudoalleles (starred, Table 1). These will be referred to as "garnet reversions." Several questions concerning the "garnet reversions" are pertinent, and further experiments were carried out with these materials. The following questions were asked:

1. Do the "garnet reversions" truly represent changes at the garnet segment of the X chromosome, or might these reversions involve mutation at (some other) locus in the form of a suppressor of garnet?

2. Other investigators, studying radiation-induced[7] and spontaneous[8] visible mutation, have recovered mutations from $g^+ \rightarrow g^x$. Among the garnets recovered, there have been several subliminal or wild-type isoalleles. These mutants are wild type when homozygous but exhibit a garnet phenotype when heterozygous with g^2 and certain other garnet mutants. Should the "garnet reversions" be due to changes within the garnet segment, might they be partial reversions similar to the wild-type isoalleles?

Each of the seventeen exceptional non-garnet individuals recovered from the crosses were subjected to the same breeding tests indicated in the discussion of the true recombinants. Exceptional males were crossed to attached-X females, and exceptional females were crossed to wild-type, Oregon-R males. In no case was there any indication of an independent suppressor of garnet. From these crosses, the "garnet-reversion" chromosomes were identified and recovered in male offspring and subjected to the following series of breeding experiments:

g-reverted; +/+ ♂♂ × +/+; st/st ♀♀

↙

F_1 g-reverted/+; st/+ ♀♀ × +; st/st ♂♂

Should g-reverted be due to a linked suppressor of garnet, one would expect that garnet males would arise among the backcross progeny. Approximately 1,000 backcross males were examined for each reversion, and no garnet flies were ever found. These observations indicate that the "garnet reversions" do not involve suppressor mutation, with the possible exception of a suppressor very closely linked to garnet.

Among the male progeny homozygous for the autosomal recessive, scarlet (st), $^1/_2$ should possess the garnet reversion X-chromosome. Since garnet modifies the scarlet phenotype, it might be possible to distinguish a partial reversion of garnet in such compounds with scarlet. The results were similar in all cases. The scarlet-appearing males among the backcross progeny were quite uniform and were indistinguishable from the scarlet stock. A second test for partial reversion is possible by examination of g-reverted/g^2 heterozygotes. Should the g-reverted chromosomes carry a subliminal garnet, then these heterozygotes should exhibit a garnet

phenotype. Each of the g-reverted chromosomes was tested and found to produce wild-type eye pigmentation in heterozygotes with g^2.

From these observations, it is seen that (1) the origin of the garnet reversions does not involve suppressor mutation but is concerned with an alteration within the garnet segment of the X chromosome and (2) the garnet reversions produce a phenotype indistinguishable from that produced by the wild type and thus constitute complete reversions rather than partial reversions.

The next question of interest is further concerned with the nature of the process giving rise to the garnet reversions. Are the reversions the result of a random process such as spontaneous reverse mutation, or is their origin in some way associated with the specific genotypes from which they segregated?

Four of the six garnet reversions arose in heterozygous genotypes involving the mutant g^{50e}. Spontaneous reverse mutation, $g^{50e} \rightarrow g^+$, with an estimated frequency, $q = 1/32{,}692$, might explain the origin of these aberrant chromosomes (Table 1). The remaining two garnet reversions segregated from the heterozygote s g^3 +/+ g^2 pl and may have involved spontaneous reverse mutation from $g^3 \rightarrow g^+$ with an estimated frequency, $q = 1/45{,}275$. Should spontaneous reverse mutation be the mode of origin of the garnet reversions, then such events would be expected to occur in all genotypes, including homozygotes, with similar low frequencies. The probability of occurrence of no reversions (α), by chance alone, in a sample of size (n), with a reverse mutation probability (q), is expressed by the first term of the Poisson distribution, $\alpha = e^{-nq}$.

That spontaneous reverse mutation is not the mode of origin of the garnet reversions is indicated by the data of Table 2. Here are tabulated assays of g^{50e} and g^3 chromosomes segregating from homozygotes and the one heterozygote which has failed to yield recombination. The heterozygote g/g^3 was assayed in both free-X and attached-X females, and both are included in Table 2.

TABLE 2

TEST OF REVERSE MUTATION HYPOTHESIS

Genotype	Event	Expected Frequency	Observed Frequency	P
g^{50e}/g^{50e}	$g^{50e} \rightarrow g+$	1/32,692	0/98,306	<0.05
g^3/g^3	$g^3 \rightarrow g+$	1/45,275	0/36,262	
dy g pl/g^3	$g^3 \rightarrow g+$	1/45,275	0/93,243	<0.05
dy g pl/s g^3	$g^3 \rightarrow g+$	1/45,275	0/11,388	
Pooled data	g^{50e} or $g^3 \rightarrow g+$	1/36,887	0/238,929	≤ 0.0015

The garnet mutants of *D. melanogaster* constitute the tightest-linked cluster of physiologically related mutants thus far to yield to recombination in this organism. The distance between $g^{50e} \rightarrow g$ (Table 1) may be used as an estimate of the lower limit of spatial resolution afforded by recombination analysis in *Drosophila*. From the first term of the Poisson distribution, it is possible to determine, for any given probability level, the number of successive failures of recombination to be assayed in order to permit the conclusion that two mutants are spatially inseparable. At the .05 level of significance, this sample number is 123,710. The only case of failure of recombination which meets this criterion is that of g and g^3 reported in this communication. With this one exception, there exists no substantial body of evidence dealing with genic structure in *Drosophila* that is in conflict with the notion of genic structure inferred from microbial studies.

Mechanisms for the origin of aberrant segregations associated with studies of limited genetic regions have been proposed by several authors.[9–12] One mechanism,[12] which suggests that the aberrant segregants arose by means of unequal crossing over in a duplicated (or further replicated) segment of genetic material, finds no support in these data. Under such a scheme, one would expect the recovery of wild types to be associated with a specific pattern of marker recombination not found in this study. These data do not permit further distinction between the various hypotheses.

Although aberrant segregations have been reported in studies of several pseudoallelic systems in micro-organisms, no similar cases have been reported heretofore in *Drosophila*. However, the similarity of the present study to the pattern of effects noted in association with heterozygotes involving reddish-alpha in *D. virilis*[6] suggests a similar interpretation for this latter system.

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SYMPOSIUM ON PLANT TUMORS

INTRODUCTION

By A. J. Riker, *Chairman*

This symposium on plant tumors serves to commemorate the fiftieth anniversary of the published description of *Bacterium tumefaciens* Smith and Town, the cause of crown gall. This disease derives its name from the relatively soft, rapidly growing gall which commonly occurs in nature approximately at the ground level or at the "crown" of the plant. The disease has important economic aspects for apples, peaches, plums, raspberries, roses, sugar beets, and many other valuable hosts. However, the economic importance is greatly overshadowed by the opportunities which this and related diseases have provided for fundamental studies on diseased growth.

Following the description of crown gall and its causal bacterium, Erwin F. Smith was much impressed with certain similarities between crown gall and tumors or cancers. His studies are described in extensive publications. More recent investigators have been equally impressed by the opportunity for fundamental work. They have applied modern techniques to the problem. They have taken advantage of the unusually favorable material provided by crown gall and comparable diseased growths. Plant tumors have some experimental facilities that are worth listing.

1. *Large Numbers.*—The ease of handling plants permits the use of many individuals. Ordinarily, the number of plants in an experiment is determined by the statistical significance desired.

2. *Valuable Experimental Procedures.*—Both the fruiting and the vegetative stages of plants can be regulated by changes in the length of day and temperature. Furthermore, certain critical physiological and even morphological changes are manipulated by alterations in the nutritional environment. Many kinds of tumors, galls, or other diseased growths are induced at will by chemical and physical agents as well as by biological agents, including viruses, bacteria, fungi, nematodes, and insects. The tumor-inducing ability of the crown-gall bacteria can be reduced by cultivation with glycine and increased by ultraviolet irradiation.

Both virulent and attenuated cultures of crown-gall bacteria are being used. Crown gall on tomato, for example, grows well below 28° C. but not above 30° C., even though the tomato is uninhibited. Crown gall can be started with the bacteria in periwinkle. Then the bacteria can be killed by heat treatment, and the tumor continues more or less autonomously, depending on the time the bacteria operate. Probably other heat-tolerant plants would serve as well.

3. *Genetic Purity.*—Many economic plants have already been developed into pure lines. However, by means of vegetative propagations, genetic variation is eliminated. For example, cobbler potatoes, Latham raspberries, and many other varieties of important crops are reproduced vegetatively, and so each individual is genetically identical with all the others.

4. *Tissue Culture.*—Animal tissues were cultivated in vitro many years ago and have contributed many techniques valuable for plant tissue cultures. These have one distinct experimental advantage, namely, unlimited active growth on a me-

dium containing only nutrients with known chemical formulas—basically nitrate for nitrogen, sucrose for carbon, and balanced mineral salts. While they are not unique advantages, perhaps one should mention that plant tissues often grow without any change of nutrient for a month or more and that the amount of growth commonly is measured by weighing the tissue. Valuable for these tumor studies are the changes induced in certain normal cultures by interactions of auxins, kinins, and other metabolic substances. The characteristics of the callus and tumorous growths, as well as the development of roots and of stems and leaves, are influenced by varying minute concentrations. Closer examinations of single factors and of interactions are possible than could be made so far with intact plants.

It is particularly fitting that this topic should be on a program where the hosts are the New York Botanical Garden and the Rockefeller Institute. Men at both places have a long history of important fundamental biological studies. Furthermore, all but one of the speakers on this program have been or are now with one or the other of these great institutions.

A TREE TUMOR OF UNKNOWN ORIGIN

By Philip R. White

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Tumors are growths of plants, animals, or men in which the normal processes of control are, for some reason, ineffective, so that continued cell division results in massive disorganized development. When they are localized and not seriously inimical to the general functioning of the supporting body, they are called "benign." When their location or manner of development is such as to kill the supporting individual, they are called "malignant" and in man become "cancers."

Tumors may arise from many causes, including parasites and infections of many kinds. The term "cancer," however, is generally limited to malignant tumors which arise from no clearly recognizable cause and in which some one or more cells of the host body have undergone a permanent change which renders them unaffected by normal growth restraints so that the altered cell itself becomes a parasite or an infectious agent.

Tumors exist in all classes of multicellular, organized living beings. In plants, essentially malignant tumors are known to be caused by hereditary factors, by certain bacteria, and by viruses.[1] Today's discussion centers around one group of such tumors of plants, the bacterial "crown gall." My own contribution deals with a massive tumor which is not known to be a "crown gall" and, in fact, appears not to be such, yet possesses features which seem to justify careful study (Fig. 1).

"Burls" are well-known massive intumescences which occur occasionally on all sorts of trees. The best-known example is the Circassian walnut burl from which finely figured veneers for cabinet work are cut. This and many other burls are probably crown galls, having the typical irregular, fungating growth pattern resulting from shifting concentrations of bacteria in the growing layers (Fig. 2). Such burls usually occur on scattered individuals, seldom in epiphytotic numbers. Trees of the White Spruce, *Picea glauca*, however, in certain sharply limited areas, are

subject to an intumescence which occurs in such numbers as to be clearly epiphytotic and which differs in structure so markedly from the usual burls that it seems doubtful whether they are crown galls.[2]

These tumors are smooth, round growths (Fig. 2). When very small, a few millimeters long on young twigs, they appear like a half-grain of rice (Fig. 4). From this they may develop into globose structures a meter or more in diameter. They occur on twigs, branches, trunks, and roots and may be single, or there may be thousands on one tree. Sections show that, irrespective of size, they *always* extend all the way to the pith (Figs. 3, 5) and hence must always arise in the bud, never from later secondary cambium.[2] On old stems a small external tumor represents the base of a narrow, acute cone of altered wood, while a large tumor represents the corresponding base of a broad, obtuse cone (Fig. 3). Each such cone is initiated by a *single cell* (Fig. 5), and the tumor trace may consist of a single file of recognizably distinct cells for 1, 2, 5, or even 20 years before it begins to expand laterally. Such files doubtless often die out and leave no external overgrowths. While these initials are always single cells, it is a common experience to find several such initials within a given region (Fig. 5), not contiguous but scattered over an area of several square millimeters of what was, at the time of initiation, procambial tissue. These intumescences thus appear to be, in fact, sectorial chimeras, arising by what seems to be a series of local somatic mutations having some common cause.

FIG. 1.—A typical rather heavily affected tree. This tree had tumors on exposed roots at distances of 6 feet and more from the base, as well as small ones on twigs and branches. It was one tree of a group of about 30 tumor-bearing individuals, about 200 feet from the shore, and in a stand mixed with white birch and Arbor Vitae, indicating a high water table.

The belief that these are sectorial chimeras of mutagenic origin is strenghtened by two physiological characteristics. First, tumor sectors are microscopically recognizable by somewhat thinner cell walls and hence slightly larger lumens; yet

the cell *size* is not sufficiently different from that of adjacent normal wood to account for the massive character of the external growths. The overgrowth arises from differences not in cell size but in cell number; a growth ring of tumor wood, in an old tumor, may have ten times as many cells as the adjacent normal wood (Figs. 6, 7). This difference in number appears to result from a greatly prolonged growth season; there is evidence that the tumors are growing actively through 8–9 months of the year as compared to about 2 months for normal wood. Since growth is dependent on a suitable combination of temperature, light, soil moisture,

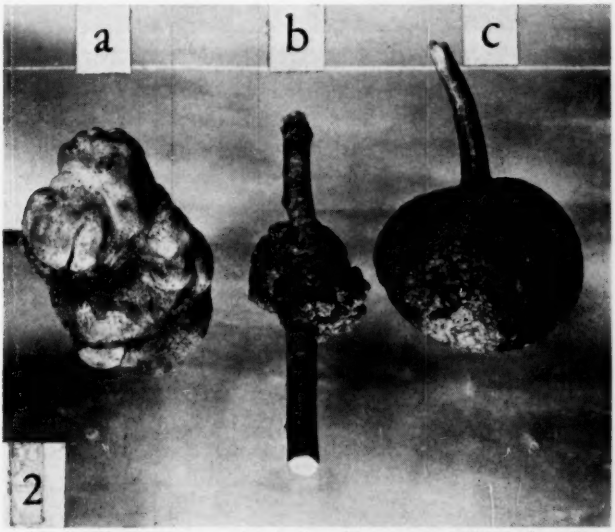

FIG. 2.—Tree tumors: *a*, beech; *b*, oak; *c*, spruce. Note that those on beech and oak have the irregular, fungating type of growth indicative of constantly shifting centers of stimulation. This is characteristic of fungus or bacterial infections and leads to the belief that these are "crown galls." The one on spruce, on the other hand, is smooth, indicating a single initial stimulus which is subsequently uniformly applied to all tumor tissue or, more probably, has become characteristic of all tumor cells.

and atmospheric humidity, of which temperature must be the most consistent variable, it seems possible that tumor cells differ physiologically from normal cells in their ability to grow well at lower temperatures than do their normal counterparts. This has not yet been tested experimentally.

Second, tissue cultures isolated from tumors are much more variable in rate of growth, growth pattern, color, and nutrient requirements than are corresponding cultures from normal wood, either adjacent to tumor wood on tumor-bearing trees or from trees which do not bear tumors. It thus appears that tumors are made up of cells which are physiologically different from normal cells both in their basic characteristics and in the instability which they show with respect to these characteristics.[3,4]

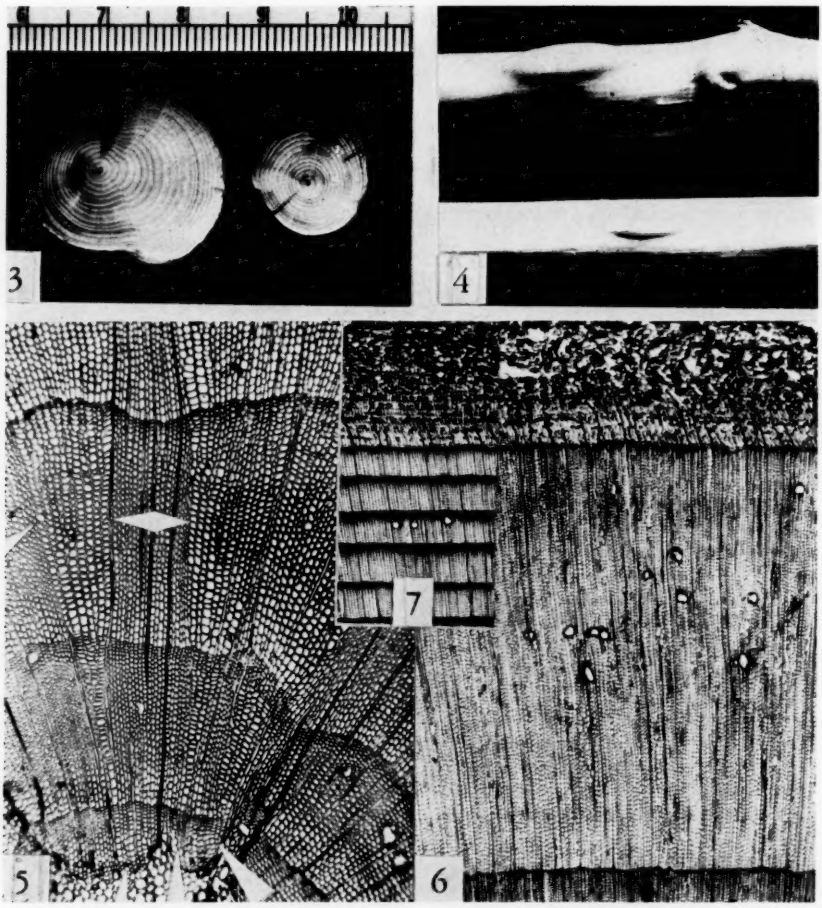

FIG. 3.—Cross-sections of two tumors from different parts of a single twig. Both can be traced to the immediate neighborhood of the pith and are hence of the same age. The difference in size is entirely attributable to different rates of *lateral* expansion, that is, to differences in the orientation of mitoses.

FIG. 4.—Examples of very small tumors. The lower one is about 6 years old. Tumors smaller than this are not externally visible under the bark of a twig but have been found sometimes (not always) by peeling twigs which show the smooth scars (absence of leaf-pegs) left after sloughing of aphid galls. Such galls live for only 1 year but may persist as dead masses for 2 or more years, leaving characteristic scars.

FIG. 5–7.—Fig. 5: A cross-section through a typical tumor. Here are three separate initials. Those at left and center developed as single files of cells for a year and a half before beginning to spread out laterally, while that at the right, adjacent to a leaf-trace, began to expand almost from the start. All three ultimately fused (beyond the area shown) to form a single intumescence. Figs. 6, 7: Sections from a tumor (Fig. 6) and from adjacent normal wood on the same stem (Fig. 7), showing the great difference in the width of the annual rings in the two regions. Note, however, that the cells in the two do not differ greatly in size, the difference resulting largely from greater cell number, due, at least in part, to the greatly prolonged growth period of tumor wood.

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As previously stated, this tumor is not generally distributed in the host species but is epiphytotic in certain clearly limited regions. It occurs on trees of *Picea glauca* growing close to the seashore, especially on exposed islands and headlands along the North Atlantic seaboard from Booth Bay Harbor, Maine, to Labrador.[2] It is not found on the other two species of *Picea* native to this region, *P. rubens* and *P. nigra*. It occurs in similar regions of the North Pacific seaboard from Puget Sound to Alaska on *P. glauca* and on the closely related *P. sitchensis* but not on the inland species, *P. engelmanni*. Yet it is also reported inland on *P. glauca* in certain limited areas in the Canadian Rockies, specifically near sulfur springs, and along the north shore of Lake Superior. Affected trees are always in groups, never isolated individuals; yet such groups always include many, usually a majority, of unaffected trees. A similar tumor is reported, without information as to host species, from certain lake regions in Germany and from the French Mediterranean coast. All these areas appear to be alike in having a high water table but have no other feature so far recognized in common.

These tumors are certainly not *typical* crown galls, either in their smooth, rather than fungating, growth habit or in bearing no bacteria capable of isolation. Yet we know that crown gall is often characterized by a relatively brief sojourn of the infection, which nevertheless results in a permanent change in cell behavior, and the production of continuously growing, yet *sterile*, tumors. It is possible that the cellular change responsible for these spruce tumors might be the result of such a transient infection, restricted to the bud stage.

They are certainly not *typical* insect galls, which in no known case continue to grow after the death or departure of the insect itself. Yet the smallest tumors we have recognized have been found under the bare scars left by the sloughing-off of galls induced by the spruce-gall aphid, *Chermes abietinis*. The female of this aphid settles on the bud in the fall, inserts its suctorium into the procambial region—precisely the region in which tumors do arise—and remains in that position throughout the winter. Such an insect might thus serve either as a primary irritating agent or as a vector for an as yet undiscovered virus or as a vector for crown-gall bacteria. Or it might provide the wound needed to "trip" a genetic lesion or provide a port of entry for some chemical agent. None of these possibilities is either excluded or demonstrated by our evidence to date.

They are not *typical* irritation teratomas, as are many burls; yet their high incidence on exposed headlands with negligible numbers on nearby protected trees has strongly suggested that salt spray or salt ground water or, in the Canadian Rockies, sulfur water or other water of low oxygen content might be a major factor in their origin. This again is unproved.

Whatever their origin, they appear to offer many reasons for further study. Their epiphytotic occurrence suggests a common, hence identifiable, cause, comparable to that of the scrotal tumors of British chimney sweeps, from whose study has come our entire knowledge of chemical carcinogenesis. The woody structure of the host, with its clearly defined annual rings, permits the tracing of each tumor to its single cell of origin and enables us to date this origin so exactly that we can define rather precisely the conditions under which the tumefacient change has occurred. This we cannot do with any animal or human tumor. Their massive character permits the isolation of parallel clones of cells from adjacent normal and

tumor sectors of a single individual and the physiological comparison of these cell lines in tissue cultures. While this is theoretically possible in animal and human tumors, it is fraught with such technical difficulties that it has not yet been accomplished over more than very brief periods, whereas in these spruce tumors it can be done with relative ease.

We believe that these tumors represent a promising addition to our roster of materials in which the processes of tumefaction can be profitably studied.

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*A PHYSIOLOGICAL BASIS FOR AUTONOMOUS GROWTH OF THE CROWN-GALL TUMOR CELL**

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We are commemorating on this occasion the description of a rather remarkable micro-organism. This bacterium, which is now known as *Agrobacterium tumefaciens* (Smith and Town.) Conn, is responsible for the initiation of the non-self-limiting neoplastic disease of plants known as crown gall. The isolation and characterization of a tumor-inducing bacterium shortly after the turn of the century attracted considerable interest among pathologists generally because at the time of that discovery no animal tumor had yet been produced experimentally. For a period of about twenty years Erwin F. Smith made detailed comparative studies of crown-gall and malignant animal tumors and found that these two types of growth had much in common. There appeared, however, to be one fundamental difference. Smith believed that the continued unregulated proliferation of the crown-gall tumor cell was dependent upon continued stimulation by the inciting bacterium. Crown gall was, therefore, not generally accepted by oncologists as being comparable to true animal tumors because, as described by Smith, this plant disease appeared to be simply a bacterial-stimulated hyperplasia and not a truly independent growth, as are most animal cancers.

In certain plant species such as the sunflower and Paris daisy there may be produced, in addition to a primary crown-gall tumor, secondary tumors that arise at points distant from the seat of the primary growth. These secondary tumors are interesting because they are frequently free of the bacteria that initiate the primary tumor. The finding that many of the secondary tumors are bacteria-free permitted the unequivocal demonstration of the truly independent nature of the crown-gall tumor cell.[1] Sterile tissue isolated from the secondary tumors grew profusely and indefinitely on a culture medium that did not support the continued growth of normal cells of the type from which the tumor cells were derived. Small fragments of such tumor tissue implanted into a healthy host developed again into

typical crown-gall tumors. Since such sterile cells isolated not only from secondary tumors but subsequently also from primary tumors of many plant species[2] have not, in the more than ten years that they have been kept under observation, shown the slightest tendency to become less autonomous, they have generally been regarded as being permanently altered cells that reproduce true to type and against the growth of which there is no control mechanism in the host. These are the characteristics by which malignant animal cells are distinguished from healthy or merely inflammatory cells. Thus the one basic difference thought by Smith to distinguish crown-gall from malignant animal tumors has been removed.

In any analysis of a complex series of events such as those that occur during tumor formation, it is often convenient to subdivide, in so far as that is possible, the total result into a series of contributing events, each of which is essential for the consummation of the completed process. In studying these events in the crown-gall disease, two distinct phases have now been recognized.[3] In the first phase normal cells are altered to tumor cells which do not as yet develop into a neoplastic growth. Two known requirements must be satisfied to complete the inception phase. These have been termed "conditioning" and "induction."[4,5] By "conditioning" is meant that only those plant cells that have been rendered susceptible as a result of irritation accompanying a wound can be altered to tumor cells. An excellent correlation has been found to exist, moreover, between the stage in the normal wound-healing cycle in which normal cells are converted to tumor cells and the size and rate of growth of the resulting tumors.[5,6] Induction, on the other hand, refers to the actual conversion of a conditioned host cell into a crown-gall tumor cell by an as yet essentially uncharacterized tumor-inducing principle elaborated by the inciting bacteria. Induction completes the first phase of tumor formation. The second phase of tumor formation, according to this concept, is concerned with the continued unregulated and autonomous growth of the tumor cell, once the cellular alteration has been accomplished. The development of a satisfactory explanation for the continued abnormal growth of a tumor cell in the absence of any recognizable infective agent represents a very real challenge not only to students of crown gall but also to those interested in autonomous growth generally. Perhaps the most important single concept that has arisen from the plant work is that concerned with the nature of the autonomous growth of a tumor cell. It is with that aspect of the problem that we should like to concern ourselves at this time.

In order to gain insight into the nature of autonomous growth, it is necessary to understand something of the processes involved in normal growth and development. Growth in all higher animals and plants is the result either of an enlargement of the constituent cells of such organisms or of the combined processes of cell enlargement and cell division. These fundamental growth processes appear to be dependent for their development in plant cells upon specific substances that may be synthesized by plant cells themselves.[7,8] It is now possible, moreover, to delimit under fully controlled experimental conditions, with the use as a test object of certain plant-cell types, these two growth processes. When, for example, tobacco pith parenchymal cells are treated with synthetic growth substances of the auxin type such as naphthalene acetic acid, the pith cells enlarge greatly in size but do not divide.[8] It is only when a second growth factor such as 6-furfurylaminopurine,[9] or the naturally occurring equivalent of that substance,[8,10] is supplied to the pith

parenchymal cells in addition to an auxin that a profuse growth accompanied by cell division results. Without an auxin, 6-furfurylaminopurine is ineffective in encouraging either an enlargement or a division of the pith cells. These findings demonstrate that two growth substances, one of which is concerned with cell enlargement and the other with cell division, act synergistically to promote growth and cell division in tobacco pith parenchymal cells. Normal tobacco pith cells do not and cannot themselves synthesize these two growth substances, for, if they did, they would respond in the characteristic manner indicated above. Since the cellular systems responsible for the synthesis of these two growth substances appear to be solidly blocked in normal tobacco pith cells, it was of interest to learn how such cells would respond when transformed to crown-gall tumor cells. The results of these studies, which are reported in detail elsewhere, demonstrated that when healing pith cells are converted to crown-gall tumor cells, typical crown-gall tumors develop.[11] This simple experiment demonstrates that, although normal tobacco pith cells did not and could not synthesize physiologically effective concentrations of either a cell-enlargement or a cell-division factor prior to their conversion to tumor cells, both substances were synthesized in greater than regulatory amounts following alteration. If this were not true, continued growth accompanied by cell division and hence tumor formation would not have resulted in the test system used in these experiments. It is clear, therefore, that an essential difference between a normal tobacco pith cell and a crown-gall tumor cell appears to be concerned at a physiological level with the permanent activation of two growth-substance-synthesizing systems, the products of which are concerned specifically with growth accompanied by cell division. The continued production in greater than regulatory amounts of the cell-enlargement and cell-division factors by the tumor cell could account for the continued abnormal proliferation of such a cell. Subsequent studies have shown,[12,13] however, that additional metabolic systems are permanently activated during the transition from a normal cell to a fully altered, rapidly growing type of crown-gall tumor cell. It has been found that alteration of normal cells to tumor cells is a gradual but progressive process.[14] When, for example, the tumor-inducing principle responsible for inception of the crown-gall tumor is allowed to act on plant cells for only 34–36 hours before being inactivated by thermal treatment, small, very slowly growing benign growths are elicited in a host. A 50-hour exposure of cells to the action of that principle results in tumors that grow at a moderately fast rate. If the tumor-inducing principle is allowed to act for 72–96 hours before being destroyed by heat, rapidly growing, potentially malignant tumors result. It is also possible to obtain tumors showing varying degrees of neoplastic change by allowing the tumor-inducing principle associated with slightly virulent or moderately virulent strains of the crown-gall bacteria to act on host cells throughout a 4- or 5-day period. Sterile tissue isolated from the three types of tumors described above and planted on White's basic culture medium retain indefinitely their characteristic growth patterns. This is illustrated in Figure 1. Since these three types of tumors were derived from the same plant species, they were admirably suited for a study of the factors required for rapid autonomous growth. In these studies, the results of which are summarized in Figure 1, the rapidly growing, fully altered tumor cell was used as the standard. This cell type can synthesize, in optimal or near-optimal amounts all the growth factors needed for its

continued rapid abnormal growth from mineral salts and sucrose present in White's basic culture medium. The moderately fast-growing tumor cell required that the basic medium be supplemented with glutamine, meso inositol, and a cell-enlargement factor (naphthalene acetic acid) to achieve a growth rate comparable to that of the fully altered, rapidly growing type of tumor cell. The very slowly growing benign tumor cells altered in a 34-hour period required, in addition to the three compounds described above, asparagine as well as cytidylic and guanylic acids to achieve full growth. Asparagine did not, however, represent an absolute require-

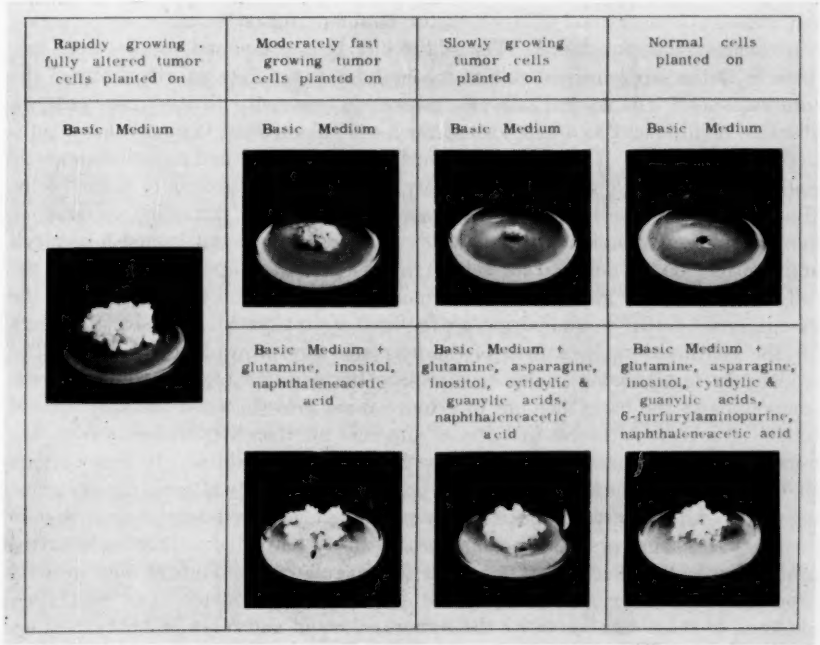
Rapidly growing . fully altered tumor cells planted on
Moderately fast growing tumor cells planted on
Slowly growing tumor cells planted on
Normal cells planted on
Basic Medium
Basic Medium
Basic Medium
Basic Medium
Basic Medium + glutamine, inositol, naphthaleneacetic acid
Basic Medium + glutamine, asparagine, inositol, cytidylic & guanylic acids, naphthaleneacetic acid
Basic Medium + glutamine, asparagine, inositol, cytidylic & guanylic acids, 6-furfurylaminopurine, naphthaleneacetic acid

FIG. 1—Relative rates of growth of three clones of crown-gall tumor tissue that show different degrees of neoplastic change, planted on White's basic medium. (*Left*), Fully altered rapidly growing tumor cells. (*Upper left*), Moderately fast-growing tumor cells. (*Upper center*), Very slowly growing tumor cells. (*Upper right*), Normal cells of the type from which the tumor cells were derived. While the three clones of the tumor cells grow continuously although at different rates on the basic culture medium, normal cells of this type do not grow on that medium. Lower pictures and legends show minimal nutritional supplements needed by the three types of tissues to achieve a growth rate comparable to that of the fully altered tumor cell. (Photographs by J. A. Carlile.)

ment for rapid growth but served to stimulate somewhat the growth of tumor tissue fragments planted on an otherwise suitable culture medium. Certain amino acids, particularly proline and histidine, also appeared to have a stimulatory effect on the growth of this tissue, but this requirement seemed to be of a transient nature, since the amino acids did not appear to be required in the second transfer of the tissue to the supplemented medium. It is clear from these experiments that, as the crown-gall tumor cell becomes more autonomous, its requirements in terms of externally supplied growth factors become less exacting. More importantly, however, these

studies clearly demonstrate that a series of well-defined growth-substance-synthesizing systems become gradually activated during the transition from the normal cell to the fully altered tumor cell, and the degree of activation of these systems determines the rate of growth of the tumor cell.

Normal cells of the type from which the tumor cells were derived do not grow on the basic medium. Thus, although the difference between the three types of tumor cells is quantitative because all grow continuously but at different rates on the basic medium, the difference between the tumor cells and the normal cell is qualitative. One qualitative difference found to exist in these studies was the absolute requirement of the normal cell for 6-furfurylaminopurine or the naturally occurring equivalent of that substance. The addition of that compound to the basic medium or to the supplemented culture media did not stimulate growth of any of the tumor tissues. The normal cells also possess, in contrast with the tumor cells, an absolute requirement as a supplement for a cell-enlargement factor such as naphthalene acetic acid. The addition of 6-furfurylaminopurine and naphthalene acetic acid to the basic medium permits the very slow but limited growth of normal cells. However, only if the basic medium is supplemented with glutamine, asparagine, inositol, and guanylic and cytidylic acids in addition to the auxin and 6-furfurylaminopurine, do the normal cells achieve a growth rate comparable to that of the fully altered, rapidly growing type of tumor cell. These same substances, with the exception of 6-furfurylaminopurine, are required as supplements to the basic medium for the continued rapid growth of the normally slow-growing tumor cells. The moderately fast-growing tumor cells require the addition of only three of these compounds to the basic medium to achieve rapid growth, while the fully altered tumor cells can synthesize in optimal amounts all their requirements from the mineral salts and sucrose present in the basic culture medium. It thus appears that, as a result of the transition from a normal cell to a fully altered, rapidly growing crown-gall tumor cell, a series of quite distinct, but well-defined, growth-substance-synthesizing systems becomes progressively activated. This leads to the production by the affected cell of greater than regulatory amounts of these growth-promoting substances. The continued production in greater than regulatory amounts of these substances by the tumor cell could and most probably does account for the continued unregulated proliferation of such a cell. Precisely how the tumor-inducing principle associated with this disease accomplishes the simultaneous unblocking of several apparently distinct and quite unrelated metabolic systems remains a moot question. These results are understandable if it is assumed that some as yet uncharacterized master reaction within the cell is specifically but gradually unblocked by the tumor-inducing principle and which, once activated, not only accomplishes the unblocking of several other growth-substance-synthesizing systems but also determines the rate at which the entire series of metabolic events concerned with growth and cell division proceeds.

The concept of growth autonomy presented above finds additional support in other directions. It has been possible to reproduce under precisely defined experimental conditions and with the use of certain normal cell types as a test object not only the morphological growth patterns[10,12] (slow and rapid disorganized growths, teratoma-like structures) but also the histological (hypertrophy and hyperplasia leading to disorganization and loss of function) as well as the cytological

(multinucleate giant cells, etc.)[15] abnormalities that characterize the tumorous state in crown gall. This was accomplished by varying the proportions of the cell-enlargement factor and the factor limiting for cell division in an otherwise suitable culture medium on which the normal cells were planted. These artificially stimulated normal cells, in contrast to crown-gall tumor cells, are self-limiting growths, and, when the externally supplied stimuli are removed, their growth promptly stops. The fact that such stimulated normal cells commonly show histological and cytological characteristics of true tumor cells but are themselves self-limiting growths indicates that the observed cellular abnormalities are the result rather than the cause of the tumorous state.

The results of all of these studies strongly suggest that it is possible for a cell to acquire the capacity for autonomous growth as a result of the permanent activation of a series of growth-substance-synthesizing systems, the products of which are concerned specifically with growth accompanied by cell division. These systems are precisely regulated in all normal plant cells.

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*ACTIVATION OF METABOLIC SYSTEMS DURING TUMOR-CELL FORMATION**

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Braun[1–4] has clearly demonstrated that the transformation of normal plant cells into crown-gall tumor cells includes the progressive activation of enzyme systems which, once in operation, become a permanent part of the metabolic machinery of the altered cells. The work in our laboratory has paralleled that in Dr. Braun's with surprisingly similar results. We have, however, utilized a different technique, permitting us, in some particulars, to garner information not readily obtainable with his system and to determine some of the interrelations between the etiological agents acting on the cells and the activation of specific metabolic systems.

A quantitative bioassay has been developed for these studies which utilizes the cambial-adjacent phloem tissues of carrot roots.[5] Tumor tissues formed in the surface of these disks can be weighed, giving a direct measure of the rates of duplication of tumor cells. We have delimited with some precision the temporal relations of the processes leading to tumor-cell formation in carrots, as well as the nature of the etiological agents (Fig. 1).

Transformation of carrot phloem cells into tumor cells occurs in three phases, designated "conditioning," "induction," and "promotion." Optimal conditioning takes 16–20 hours and requires the action of several causal agents normally present in wound juice.[2, 6, 7] The bacteria are unnecessary at this time. By pre-assay, carrot roots are obtained which are deficient in one of these agents,[7] and these roots possess only limited tumor-forming capacity. Conditioning may also be limited by restricting the time of action of these agents, by interfering with their action with antimetabolites of the conditioning agents, or by slowing down the process with cold treatment.

Induction takes 60–70 hours for completion and requires the action of a tumor-inducing principle (T-iP) which may be a DNA synthesized by genetically competent crown-gall bacteria in the presence of a T-iP synthesis factor also present in the wound juice.[8] Induction may be limited at will by using strains of crown-gall bacteria synthesizing a less effective T-iP or by holding the conditioned and inoculated disks for given periods of time during the induction phase at elevated temperatures[9] where T-iP is presumably not synthesized by crown-gall bacteria.

Incipient tumor cells formed by the action of conditioning and induction agents are promoted into primary tumor cells by auxins.[10, 11] Promotion requires 28–30 hours in carrot. This phase has been inhibited by presenting an antiauxin to the tissues during the critical time. Tumors are macroscopically evident within 12 hours after the end of transformation, and their rates of duplication are linear for 10–15 days.

For optimal transformation, the amounts, composition, and presentation times of all causal agents must be precisely regulated. Any deviation from the optimum results in the formation of slow-growing tumors. The exogenous stimulation of slow-growing tumors resulting from incomplete conditioning, induction, or promotion by specific growth factors may be taken as presumptive evidence that the

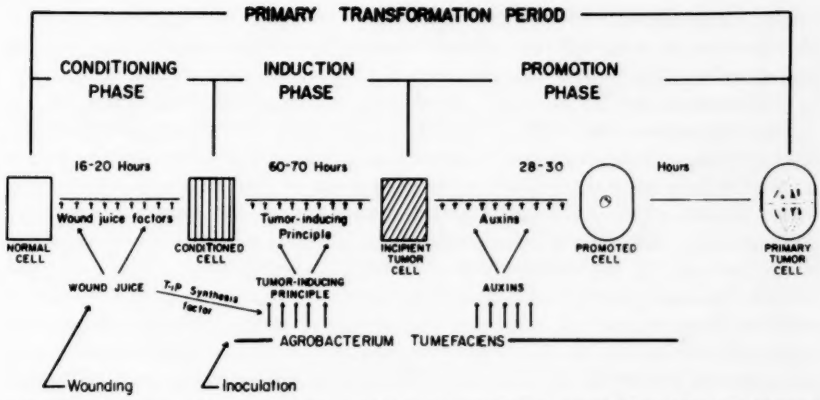

FIG. 1.—Diagrammatic representation of the transformation of normal plant cells into primary crown-gall tumor cells.

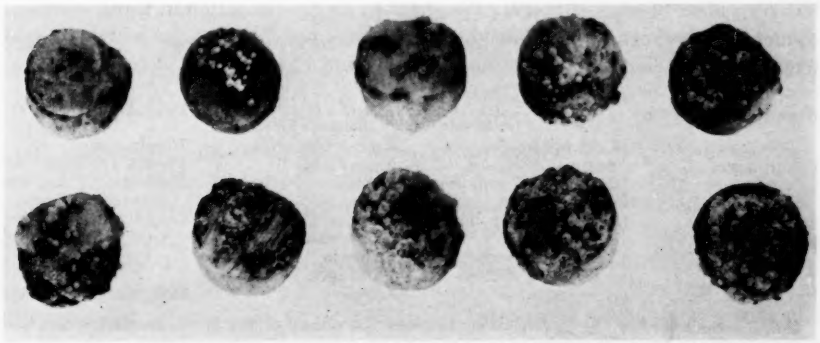

FIG. 2.—Distribution of tumor sizes following endogenous limitation of wound-juice factors required for conditioning.

metabolic system synthesizing that factor was not fully activated by the causal agents operative during the modified step. Thus analyses of the subcellular consequences of transformation are feasible.

The procedure used was as follows. Disks of cambial-adjacent carrot phloem were exposed to the agents acting in the three steps of transformation, and one of these steps was modified. At the end of the transformation period, the disks were transferred to Petri dishes containing water agar plus the test compound, and, after a period of growth, the tumors were weighed. Figure 2 shows a series of tumors resulting from endogenous limitations of one of the conditioning agents. There is a complete spectrum of tumorous growth capacities ranging from virtually no duplication to relatively massive and confluent tumorous development. Similar pictures could be presented following incomplete induction or promotion.

The significant findings to date are presented in Figure 3. We have found that, following incomplete transformation, three biochemical categories of slow-growing

tumors are produced, each characterized by its own set of nutritional deficiencies.[12] Slow-growing or weak tumors, formed following incomplete conditioning, can be stimulated only by purine ribosides, several amino acids, and one or more of the intermediates of the Krebs cycle. Weak tumors formed following incomplete induction require mevalonic acid, gibberellic acid, kinetin, and coconut milk for maximum growth. Preliminary data indicate that the kinetin response may be due to its mimicking a natural cell-division factor found in coconut milk and in crown-gall tumor tissues. This cell-division factor is not any of the known fractions of coconut milk. The slow-growing tumors resulting from incomplete promotion are stimulated only by indoleacetic acid or its biological equivalent. It is important to note that compounds that stimulate weak tumors of one category are without positive effect on tumors from another etiological group. Finally, extracts of crown-gall tissues contain all the substances which positively affect the growth of slow-growing tumors of any category. The positive effects of the required growth factors are observed only with incompletely activated tumors, and the increased growth is apparent only as long as the required factor is available. The stimulation is temporary, not permanent. The failure of these substances to activate the system permanently when supplied subsequent to its time of action in transformation cannot be experimentally explained, although it is possible that the metabolic systems of tumor cells are not susceptible to activation.

Phase of Transformation Period	Technique Used to Render Phase Incomplete	Incompletely Activated Synthesis Systems	Metabolic Systems Affected
Conditioning	Cold treatment	Krebs cycle acids	Respiration
	Antimetabolites	Amino acids	Protein synthesis
	Quantity of conditioning agents	Purines	Nucleic acid and coenzyme synthesis
Induction	Bacteria of varying virulence	Mevalonic acid Gibberellins	Isoprenoid synthesis
	Temperature inhibition of T-iP synthesis	"Kinetin" and "Coconut milk factor"	
Promotion	Antiauxins	Auxins	Cell enlargement, etc.

FIG. 3.—Summary of the interrelations between the phases of tumor-cell formation and the metabolic systems activated during transformation.

Even within the relatively short time of induction, there is evidence that the activation of different metabolic systems occurs independently and in a given order. Thus mevalonic acid prototrophy is found within 24–48 hours, while kinetin and gibberellic acid independence requires 48–72 hours. Exact correspondence of data between two different plant systems cannot be expected. Pyrimidines and vitamins were not required by slow-growing carrot tumors of any category but were required by *Vinca*,[4] and *Vinca* tumors do not seem to require mevalonic acid or organic acids. Apparently, different metabolic systems are either already developed in healthy cells of various plants or are not involved in the growth of the tumors derived from these cells.

The interrelation between the chemical nature of the etiological agent and that of the growth factor synthesized by the activated metabolic system is quite intriguing. A purine and possibly amino acids are required for conditioning, and we may assume that purine- and amino acid–synthesizing systems are developed. An identical situation holds for auxins in promotion. In crown gall, etiological agents apparently activate systems permanently regulating the synthesis of compounds

chemically related to the agents, a finding best explained by invoking a feedback mechanism.

The stability of the acquired synthetic systems is one of some practical importance in oncology. Since growth of plant tumors is dependent upon the activation of metabolic systems, their selective destruction should stop growth. We have found that the systems activated in carrot during conditioning, particularly those synthesizing amino acids and purines, are temperature-labile,[13] that visible radiation inhibits the gibberellic acid system activated during induction, and that ionizing radiation will suppress the auxin-regulating systems.[14] Further work on this concept may prove useful in the control of animal tumors.

It must be emphasized that the ability of plant tumor cells to synthesize all required growth factors is not the entire "cause" of crown gall. Morphogenic (graft) autonomy of tissues is observed following transformation periods shorter than those required for full activation of all metabolic systems,[15] and there is no evidence that certain respiratory and energy metabolism characteristics are related to growth-factor synthesis.[16]

The acquisition of the ability to synthesize any given growth factor can be treated formally and is of general biological importance.[16] Enzyme synthesis may be completely or partially prevented by a series of factors, not the least being the inherent genetic capacity of the cell. For a cell to be even potentially capable of synthesis, there must be an adequate genetic potential which can be modified only by mutation. For adequate enzymatic potential—and crown gall is a case in point—etiological agents must act on enzyme-forming sites to permit the development of the metabolic system concerned.

Finally, the synthesis of the factor requires precursors, which are made available by other metabolic systems. When all necessary conditions are fulfilled, the cell is prototrophic for the growth factor being studied. There are sufficient data in the literature to suggest that morphogenic alterations in the behavior of many cells—both plant and animal—may be so analyzed. It is for this reason that crown gall may be a useful model for growth and differentiation in cells.

Viewed historically, the crown-gall problem has proceeded in a series of decade-long steps. The first decade was concerned with Smith's struggle to convince the doubters that bacteria could indeed be the cause of plant disease. The second ended with a relatively precise structural analysis of the crown-gall syndrome, and the third found our knowledge of the chemical structure of the tissue on a firm foundation. The fourth decade was the period in which the biology and chemistry of the causal agents began to assume its present form, and the fifth, now ended, has taken the problem to the intracellular level discussed today. Research on crown gall has, of necessity, been on the frontiers of current knowledge in cellular biology and has been both a recipient and a donor of knowledge on this frontier. This situation will, happily, continue.

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STIMULATION OR INHIBITION OF VIRUS OF INFECTED AND INSECT-GALL TISSUES AND SINGLE-CELL CLONES

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Plant tissue culture has provided an important method for clarifying normal and diseased growth. The method has been used in the present studies with tissues from a variety of plant species to study stimulation and inhibition of diseased growth of crown gall, virus, and insect-gall origins and the normal homologous tissues from the same species. Recent success in culturing for unlimited periods clones of tissue established from hand-picked single cells of the diseased and normal tissue masses[1] provided another means for elucidation of similarities and differences among the cells in the tissues.

The early independent works of Kotte, of Robbins, and of White showed the possibility of the in vitro growth of isolated root tips.[2] Their successes indicated the need for the proper balance of inorganic salts, carbohydrates, and growth substances to culture isolated plant tissues successfully. These early studies led to the development of true tissue or cell cultures, respectively, by White, by Gautheret, and by Nobecourt.[2] Certain of these tissues and others from roots, stems, and leaves have been described in this symposium as tools for studying the specialized problems of differences between normal and diseased growths.

In order to understand these diseased conditions better, many other studies have been made with isolated tissues that grew in vitro. These came from crown galls, virus-infected tissue, and insect galls. The results of some of these studies are described below.

Tissues of Crown Gall and Normal Origins.—The tissue for these studies came from cultures established from 5 to 16 years previously. They were transferred aseptically to fresh medium[3] at regular intervals as needed. All experimental cultures were started by transferring good stock tissue to a sterile Petri plate, cutting it with a sterile scalpel into seed pieces 20 mg. in mass and by transferring four good pieces with a sterile wire loop to each experimental culture bottle. Replications were usually six culture bottles for each experimental condition.

With these tissue cultures, it was possible further to clarify the balance of inorganic salts, sugars, alcohols, organic acids, and various nitrogen compounds re-

quired for the best growth of these tissues of gall and normal origins.[4] The results provided a background needed to define important nutritional and physical environments required for growth or inhibition of growth.

Equally important as knowing the compounds favoring growth of the cultures was the inhibition of growth induced by low concentrations of common metabolites.[5] The importance has also been described of low levels of common metabolites in differentiation and growth of maturing stem tips[6] and freshly isolated callus.[7]

Interesting results also appeared with nucleic acids and their components for tissues of gall and non-gall origins.[8] When tissues were incubated on media containing each, respectively, of the component purines and pyrimidines, little effect was observed as measured by the wet weight of the gall and normall tissues. However, ribonucleic acid (RNA) stimulated growth of both tissues, while deoxyribonucleic acid (DNA) had little effect or inhibited growth. The stimulation and inhibition of different levels were of additional interest because the tobacco mosaic virus is largely RNA. Different concentrations of virus may, therefore, influence stimulation or inhibition of host growth in relation to the RNA it provides in the cell.

Virus-infected Plant Tissue Cultures.—Virus-infected tissue cultures have been studied to clarify the conditions stimulating or inhibiting the multiplication and infectivity of the virus.[9–12] The tobacco tissue (*nicotiana tobacum*) of crowngall origin and containing the tobacco mosaic virus was originally isolated and supplied by Morel.[13] The appearance of the established cultures is shown in Figure 1. This tissue had considerable virus (0.1 mg/ml of sap). It was subcultured at regular monthly intervals for over 8 years, with no apparent change in the virus concentration as measured by the standard local-lesion assay method on half-leaves of the local-lesion assay plant (*N. tabacum* × *N. glutinosa*). The amount of virus and infectivity per unit wet weight of tissue was indicated by counting the number of local lesions produced by a homogenate of the infected tissue cultures.

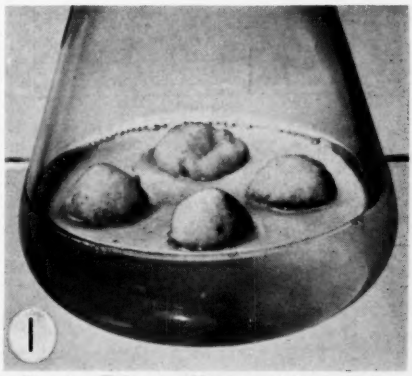

FIG. 1.—Tobacco (*Nicotiana tabacum*) tissue of crown-gall origin, free of the inciting bacterium but infected with tobacco mosaic virus. Four tissues after 5 weeks' incubation as they grew on a synthetic medium in 125-ml. Erlenmeyer flask.

Early studies[11] indicated that temperature influenced the amount of host growth and the amount of virus present. The growth of the host tissue, expressed as the average wet weight after 6 weeks' incubation, and the concentration of the virus, expressed as the average number of local lesions per half-leaf, are seen in Figure 2. Greatest host growth developed on media incubated over the temperature range 20°–36° C., with the optimum at 26° C. The decrease in growth at 28° C. was not clear. Perhaps it was correlated with the decreased virus concentration initiated at 28° C., shown in the lower portion of the figure. It was of special interest that a temperature differential existed for optimum host growth and for virus multi-

plication. The virus concentration was much reduced per unit mass in tissues incubated at 36° and 37° C. Inactivators of virus synthesis were suggested by reduced local lesions from cultures incubated at from 28° to 37° C. where good host growth developed. Virus activity per unit weight was at a normal level in cultures incubated at temperatures of 38°–42° C. where the host tissue was killed.

The importance also was indicated of the acidity[11] of the medium for host tissue growth (Fig. 3). The tissue grew more or less on media with final acidities of pH 4.5–8.5; optimally at pH 5.0–7.5; and failed to grow from pH 2.0 to 4.0 or from 9.0 to 11.5. The infectivity of the virus from the tissues assayed on the local-lesion host at weekly intervals during the 6-week incubation period showed the most virus in tissues incubated on media with acidities from pH 8.0 to 8.5 (Fig. 4). Little or no virus was present in tissues incubated on media at pH 9.3–9.5 during the 6 weekly assays. The assays showed that virus multiplication was most rapid during the first 3-week period. Also during the first 3 weeks the differences were greater between amounts of virus in tissues incubated at the different respective acidities than during the fourth, fifth, or sixth weekly assays.

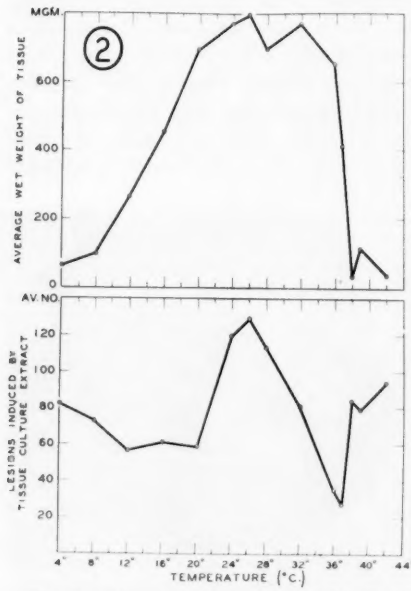

FIG. 2.—*Above:* Average wet weight after 6 weeks' incubation at temperatures indicated of tobacco tissue infected with tobacco mosaic virus. *Below:* Average number of local lesions per half-leaf produced on hybrid tobacco assay plant by tissue homogenate from cultures incubated at respective indicated temperatures.

Different levels of nitrogen, phosphate, and potassium[10] in the medium stimulated or inhibited tobacco mosaic virus and host tissue growth, depending on the concentration. The effects of increased levels of NaH_2PO_4 (for phosphate) appear in Figure 5. In contrast to increased nitrate levels (Fig. 6), the virus activity decreased per unit weight with increasing concentrations of phosphate. With different levels of $NaNO_3$ (for nitrogen) (Fig. 6), growth developed poorly without $NaNO_3$, best with 0.4 or 1.6 gm/liter, and was inhibited by 25.6 gm/liter. The virus activity in the tissues increased with increasing levels of $NaNO_3$ to 1.6 or 6.4 gm/liter. Even with 25.6 gm/liter, where host tissue growth was inhibited, the virus activity remained high. The virus activity was influenced modestly by increasing levels of KCl (for potassium) (Fig. 7). A moderately greater virus activity developed in cultures on media with progressively increased levels up to 6.4 gm/liter of KCl. Host tissue growth, however, was much improved by the addition of KCl that corresponded closely to that in the basal medium.

The amounts of host tissue growth and virus infectivity were influenced by the kind and amount of nitrogen present. An extensive series of tests[12] were made

where each, respectively, of different common nitrogen compounds were tested over a range of concentrations comparable to that with $NaNO_3$. These experiments indicated that the tissue was inhibited more or less with all concentrations of $NaNO_2$, NH_4Cl, $(NH_4)_2SO_4$, ammonium lactate, or ammonium tartrate. NH_4NO_3 and ammonium succinate stimulated tissue growth at median levels. The infec-

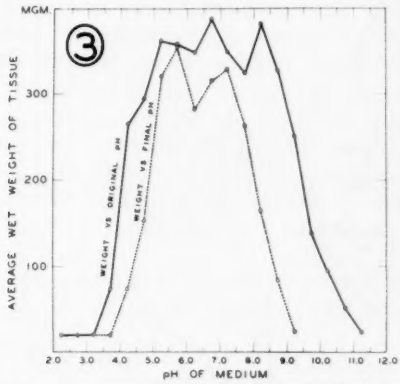

FIG. 3.—Average net weight of TMV-infected tobacco tissue after 6 weeks' incubation on basal medium with respective acidities as indicated.

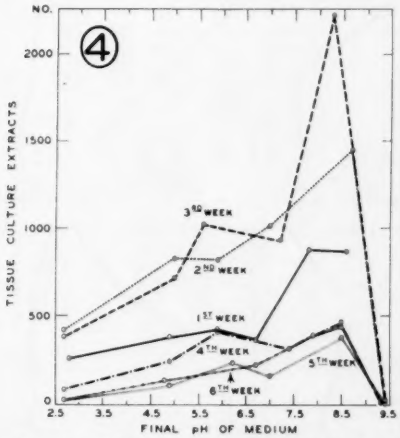

FIG. 4.—Tobacco mosaic virus infectivity of homogenate of tobacco tissue cultures incubated on media with acidities as indicated and assayed at 6-weekly intervals.

tivity of the virus per unit weight of wet tissue increased over controls in cultures with 0.1 and 0.4 gm/liter $NaNO_2$; 1.6, 6.4, and 25.6 gm/liter NH_4Cl; 1.6 gm/liter $(NH_4)_2SO_4$; 1.28, 5.12, or 20.48 gm/liter NH_4NO_3; 0.4, 1.6, and 6.4 gm/liter ammonium succinate; 1.6 and 6.4 gm/liter ammonium tartrate; and none with ammonium lactate. Provided that the host tissue grew at all, the virus multiplied

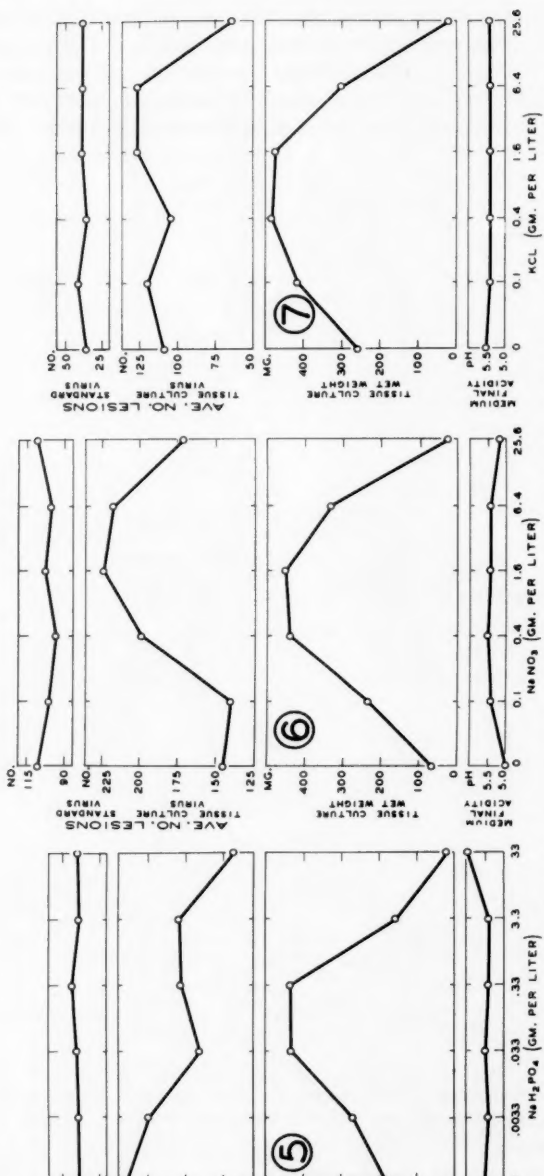

Fig. 5.—Effect of increased concentration of NaH_2PO_4 on host tissue growth after 6 weeks' incubation expressed as the average wet weight in milligrams. The virus activity is expressed as the average number of local lesions produced in half-leaf, local-lesion assay by a homogenate on a unit-weight basis of the virus-infected tissue from the respective media. The average number of local lesions produced by a dilution of standard TMV on opposite half-leaves is indicated at top. The final pH of the respective media is plotted at the bottom.

FIGS. 6, 7.—Same as Fig. 5, except at different levels, respectively, of $NaNO_3$ and KCl.

with increasing concentrations of nitrogen. Only when host tissue growth was inhibited was the virus infectivity greatly reduced.

Recently the influence of concentrations of nucleic acids, purines, and pyrimidines and some of their analogues was studied. The concentration effects of certain of these compounds on the growth of the host tissue and on the virus activity in the tissues are summarized in Table 1. All these materials more or less influenced cellular growth as well as virus multiplication, depending on the concentration. Except for thymine and thiouracil, which progressively inhibited growth with increased concentrations, the other compounds stimulated the host tissue more or less at specific concentrations. Except for thymine and thiouracil, the compounds resulted in increased virus activity in the tissues. Increased levels of uracil were most beneficial for virus multiplication. Thymine was especially interesting because it reduced host growth only about 30 per cent at the highest level but reduced virus activity 17 per cent. Thiouracil was the most inhibitory to virus multiplication, but it also essentially stopped the host tissue growth. Some of these results confirm related studies by others[14] who used certain of these materials and also described altered virus multiplication in tobacco mosaic virus-infected leaf disks or tissue cultures.

TABLE 1

TOBACCO MOSAIC VIRUS CONCENTRATION IN HOST TOBACCO TISSUE CULTURES GROWN ON SYNTHETIC MEDIA CONTAINING PURINES AND PYRIMIDINES

Conc. (mg/l.)	DNA W*	DNA TMV†	RNA W	RNA TMV	Adenine W	Adenine TMV	Thymine W C‡	Thymine TMV	Uracil W[1]	Uracil TMV[2]
0.....	220	37	299	40	958	72	930(0)	100	517	66
0.01..	245	37	333	42	1030	72	823(00.5)	92	528	75
0.1...	250	40	379	40	1056	69	824(0.5)	89	489	74
1.0...	255	39	295	43	943	72	752(5)	94	501	75
10.0...	423	38	339	40	849	58	764(50)	93	374	82
100.0...	216	40	227	42	872	74	636(500)	83	252	84

Conc. (mg/l.)	Thiouracil W	Thiouracil TMV	6-Methyl-Uracil W	6-Methyl-Uracil TMV	2-Thio-6-Methyl-Uracil W	2-Thio-6-Methyl-Uracil TMV	Propyl Thiouracil W	Propyl Thiouracil TMV	6-Methyl 4-oxy-2-Thiopyrimidine W	6-Methyl 4-oxy-2-Thiopyrimidine TMV	2,4-Dithio-5-Methyl Pyrimidine W	2,4-Dithio-5-Methyl Pyrimidine TMV
0.....	221	46	207	19	273	36	147	31	230	11	208	44
0.01..	190	53	203	28	296	41	160	30	230	15	198	44
0.1...	180	48	203	22	261	41	163	32	240	10	220	36
1.0...	163	42	210	23	292	43	157	32	228	13	209	44
10.0...	17	25	215	24	250	42	135	38	208	13	195	49
100.0...	47	24	195	23	204	44	66	35	180	12	163	42

* Average wet weight of 72–120 tissue pieces in milligrams.
† Average number of tobacco mosaic virus local lesions from 90–150 half-leaves. Dilution 1:2000 on a tissue culture wet-weight basis.
‡ Concentration in milligrams per liter.

Certain of these materials stimulated both host growth and virus activity. Thiouracil, however, reduced virus activity largely only where it was toxic to the host. This suggested the possibility that other compounds might exist that would inhibit virus multiplication at levels that had little or no toxicity to the host cells. First success in this direction appeared with increased levels of thymine. More recently, 6-methylpurine[15] was found especially inhibitory to virus multiplication in the host tissue at levels that were little or not at all toxic to the host tissues. These results were directly opposite to those secured with certain of the thiopyrimidines that were toxic to the host cells. In fact, concentrations of 0.01 mg/liter of 6-

methylpurine reduced virus activity 10 per cent; the host tissue was actually stimulated. Slightly higher concentrations that reduced virus activity 30 per cent just bordered on phytotoxicity. The mechanism of this selectivity of action of 6-methylpurine is of special interest.

Plant Tissue Cultures of Insect-Gall Origins.—A more recent study of gall tissues was concerned with the possibility of culturing gall tissues induced on plants by insects. The hope has been to learn the manner in which these very abnormal types of differentiation are incited by the insects and the metabolic balances required for their continued growth and development.

The background information gained by culturing crown-gall and virus-infected tissues made it possible to establish indefinite cultures from a variety of galls on willow, grape, poplar, elm, and many other hosts.[16] More recently the effects were reported of air temperature and the acidity of the culture medium on growth of these four excellently growing species.[17]

These insect-gall tissues required, for best growth, complex media containing such materials as coconut milk and 2,4-dichlorophenoxyacetic acid (2,4-D) in addition to the basic inorganic salts, sucrose, and vitamins. The importance of coconut-milk supplements for growth of certain gall and normal tissues is seen in Table 2. These data also indicated the variation in requirements between species and within species. For example, a strain of tissue isolated from a grape *Phylloxera* gall grew best with 100 ml/liter coconut milk, while tissue from a grape petiole gall grew best with 400 ml/liter or more of coconut milk.

TABLE 2

EFFECT OF COCONUT-MILK CONCENTRATION SUPPLEMENT TO BASIC MEDIUM ON GROWTH OF SOME REPRESENTATIVE INSECT-GALL AND NORMAL TISSUES IN VITRO

SUPPLEMENT TO BASAL MEDIUM OF COCONUT MILK (ML./L.)	AVERAGE WEIGHT OF TISSUES (MG.)*				
	Willow Normal	Willow Leaf Gall	Grape Normal	Grape Phylloxera	Grape Petiole Gall
0	80	70	90	100	30
25	190	180	180	340	50
50	250	260	350	700	80
100	310	360	490	980	140
200	350	520	610	960	420
400	410	650	520	460	450

* Each number represents the average wet weight in milligrams of 72 tissue pieces from three experiments.

Detailed studies were carried out[16] and are in progress to clarify the growth-substance requirements of these gall tissues provided by the balance of factors present in the coconut-milk, 2,4-D media. Certain of the gall and homologous normal tissues grew more or less on synthetic media without coconut milk but containing kinetin plus naphthaleneacetic acid. Some comparative growth results with synthetic and coconut-milk media are illustrated in Figure 8. The need for other balances of materials in these media lacking coconut milk was evident.

Tissue Culture Clones of Single-Cell Origins.—The interest, of course, in compounds or common metabolites that stimulated or inhibited cellular growth and differentiation has broad significance. The question appeared whether there could be introduced into a diseased cell or cell mass a common metabolite or another compound capable of being metabolized in the cell so that it would inhibit

the diseased cell and not injure the normal cells. Results with thymine and 6-methylpurine were encouraging in this connection.

In order to develop such a control for diseased growths, we need first to understand the balance of conditions prevalent in the diseased cells. This, in turn, raised the question whether all the cells in a gall were diseased or, in fact, even diseased to the same degree. Or were a few diseased cells responsible for a mass of abnormal, rapidly increasing cells, and the majority of the other cells providing a favorable nutritional and physical environment for the rapid multiplication of the cells? Success by Braun[18] in obtaining tissue free of crown-gall qualities by grafting to rapidly growing plants was pertinent to this question.

In the present studies, an answer to possible cell differences was approached by culturing clones of tissue started by isolating single cells from the mass of established gall or normal tissues that grew in vitro. This has been successful with a variety of animal tissues.[19,22] Various methods have also been tried to culture plant tissues from a single cell.[20] The method used here for single plant cell cultures was originally described in 1954 by Muir, Hildebrandt, and Riker.[1] The single-cell cultures in the present studies were secured under aseptic conditions by spreading liquid or agar tissue cultures on sterile agar in a Petri plate. The single isolated cells were picked up with a microspatula by hand while viewed under a dissecting microscope. The cell was then transferred to an 8-mm. square of sterile filter paper resting in the Petri plate. The filter-paper square had been placed 3–4 days previously on the established host-mother tissue cultures so that it had absorbed a favorable balance of nutrients from the culture. The filter paper raft with the single cell was then transferred back to the mother tissue. These were then, in fact, individually picked cells and not colonies of cells or dispersions of single cells.

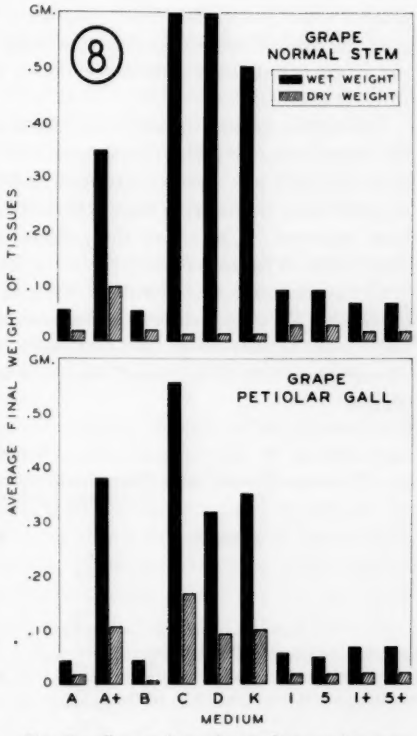

FIG. 8.—Comparison of growth on various synthetic and natural media of tissues isolated from normal stem and a petiolar gall tissue of grape. Growth was measured as the average wet or dry weight of the tissues. Growth on the basal synthetic tobacco medium A[3] containing inorganic salts, sucrose, and vitamins was compared with growth on A medium with supplements as follows: *A+* = A plus 150 ml/l coconut milk (CM); *B* = A plus 1×10^{-4} gm/l naphthaleneacetic acid (NAA); *C* = A plus CM, NAA, and 2.5 mg/l calcium pantothenate (CP); *D* = A plus CM, NAA, CP, and 6 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D); *K* = A plus CM, CP, and 2,4-D; *1* = A plus 1×10^{-4} gm/l kinetin (K); *5* = A plus 5×10^{-4} gm/l K; *1+* = A plus NAA and 1×10^{-4} K; and *5+* = A plus NAA and 5×10^{-4} gm/l K.

If the isolated single cells were placed directly on the medium supporting the

mother tissue piece, they failed to grow. However, when placed on the filter paper over the established culture, after several days to weeks, depending on the isolate, the single cell divided sufficiently that it could be transferred directly to the agar, where it grew.

During the period the single cell incubated, the growth was observed as desired by simply removing the filter-paper raft and cell from the established culture by transferring it to a sterile Petri plate and by observing under the microscope. The cell and filter paper were then returned to the culture vessel from which they had been removed. Certain of the isolated cells grew slowly, so it was necessary to transfer them to fresh mother pieces as the original cultures became senescent.

Clones of tissue thus derived from individual single cells from the tissue masses of crown-gall origin showed differences in growth rates, color, cell morphology, nutritional requirements, and texture.[21] Further studies were aimed to learn more about the ability of single-cell clones to induce galls when grafted back to healthy plants.

TABLE 3

TOBACCO MOSAIC VIRUS CONCENTRATION IN SINGLE-CELL CLONES OF TOBACCO TISSUE CULTURES INOCULATED WITH TMV

CLONE NO.		No. Local Lesions* Tissue	No. Local Lesions* Medium	Av. Tissue Weight (Gm.)†
N. tabacum × *N. glutinosa*	160	4	0	8.73
	169	3	8	10.06
	176	6	0	9.20
	194	158	7	8.56
	195	5	4	10.60
	196	265	2	9.55
	197	6	7	12.32
	237	77	6	4.10
	239	8	0	6.10
	240	5	2	7.17
	246	5	1	13.30
	261	0	0	11.90
Morel's *N. tabacum*	222	0	6	10.39
	223	0	0	2.05
	471	349	184	3.74
N. glutinosa	252	83	4	0.54
	283	146	5	0.40

* 1:1 dilution of tissue homogenate assayed after 4 monthly transfers in liquid "D" medium.
† Average weight in grams of tissues in 4 bottles after 4 weeks' incubation.

Single-cell clones isolated from tobacco tissue either free from or infected with tobacco mosaic virus (TMV) were also studied. Variations in growth of single-cell clones, in susceptibility to virus infection, and conditions stimulating or inhibiting virus multiplication were first studied. Twenty single-cell clones to tissues isolated from established tissue cultures from the local-lesion hosts (*N. glutinosa*, and *N. tabacum* × *N. glutinosa*) and two from the systemic host *N. tabacum* indicated differences in growth rate, in texture, and in their requirements for growth in liquid and on agar media. The single-cell clones may also differ in their susceptibility to infection by virus and in the multiplication of the virus in the tissues. Differences in growth rates and in the amount of TMV present in some representative clones appear in Table 3. The cultures were incubated in liquid media, to which a sterile filtered TMV preparation had been added 4 months previously to give 1:1000 dilution with the basic medium. Growth and virus activity were measured on the

basis of the wet weight of tissue after the fourth monthly transfer to fresh medium. Virus assays were made, respectively, of the tissue and the medium with the half-leaf method on the local-lesion host (*N. tabacum* × *N. glutinosa*).

Conclusions.—A great variety of important tissues and techniques are therefore available to clarify normal and diseased growth. Results already described by this panel indicate that much work has been done and considerable progress made toward understanding the host-parasite relationship resulting in disease. There is good reason to hope for further clarification of the mechanism of disease initiation and perpetuation and how it may be stopped.

Tissue cultures from a variety of diseased and normal origins were readily cultured on relatively simple, chemically defined media. The tissue came from crown galls, insect galls, and homologous normal tissues on many different plant species. Certain isolates, however, required more complex balances of chemicals present in coconut milk. The concentrations of some simple common metabolites are critical in stimulating or inhibiting growth.

These studies have further indicated that the component cells in the diseased and normal masses of tissue that grew in vitro varied considerably in morphology, physiology, and pathology. We, have, therefore, in this single-cell technique an additional tool to search for the combination of metabolic balances that distinguish a diseased cell from a healthy one. Can we so define this balance in terms of individual or groups of compounds so that certain common metabolites can be used to inhibit the diseased cells and not injure the healthy ones?

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WOUND-TUMOR

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The wound-tumor disease of plants was discovered about thirty-five years after the isolation of the specific bacterial incitant involved in crown gall. Whereas the wound-tumor disease is of no known economic importance, crown gall is a serious disease of plants, and its ravages have provided a strong incentive for its study. Nevertheless, our greatest advances in understanding crown gall derive from investigations aimed to satisfy men's curiosity rather than from those designed to control the disease. By this time many biologists are familiar with crown gall; few are acquainted with wound tumor, although it is one of that interesting group of tumors incited by viruses.

Crown gall is chiefly a local infection with a limited development of secondary tumors in a few suscepts or hosts. Wound tumor, on the other hand, is caused by a systemic virus, and in most hosts it produces systemic irregular vein enlargement and many tumors on the roots. The wound-tumor virus has been transmitted to many plant hosts experimentally. It produces its most severe root-tumor manifestations in sweet clover, *Melilotus alba* Desr. and *M. officinalis* L. Lam., and in sorrel, *Rumex acetosa* L. Noticeable stem tumors occur only in sweet clover.

Histologically, the tumor contains meristematic tissue, sometimes organized as a cambium but usually consisting of scattered, meristematic zones. It also contains distorted tracheids and an abnormal phloem-like tissue. The tumor ordinarily contains no vessels and no sieve tubes. In the roots, most of the tumors originate in the pericycle; in the stems and leaves, the overgrowths start from the procambium, parenchyma, or fibers of the phleom. In roots of shepherd's purse, *Capsella bursapastoris* (L.) Medic., lateral roots are arranged in two rows directly opposite each other and running the length of the root; the initial tumor cells also form two rows precisely located on opposite sides of the root, midway between the points of origin of the lateral roots. In other words, at any time the point of origin of the next initial tumor cell can be predicted with great accuracy.

Tumors contain many spherical inclusions which have been shown to be rich in arginine. It is not known whether or not the inclusions are rich in virus.

Sweet-clover plants vary greatly in their hereditary response to infection with the virus, some producing many prominent root tumors and others very inconspicuous ones. Because it is a simple matter to produce sweet-clover plants from cuttings, many plants belonging to a single clone and having identical genetic constitution can be readily obtained. The virus can be introduced into a number of plants in each of several genetically different clones. Such a study has demonstrated clearly that the size, number, distribution, and morphology of the tumors on the stems of sweet-clover plants are affected by the heredity of the host.

There is abundant evidence that wounds are important in initiating tumors in virus-infected plants. About 80 per cent of the root tumors originate in the immediate vicinity of the emergence points of lateral roots. Lateral roots start in the pericycle and make wounds in the overlying endodermis, cortex, and epidermis through which they penetrate to the outside. On the stems of sweet clover, tumors are most common at the point of origin of branches, which are, of course, points of

stress. They also occur at graft unions and at the cut surface of cuttings. Under optimum conditions they occur at artificially made wounds in young stems. The younger the wounded part of the virus-infected stem, the greater the probability that a tumor will develop from the wound. Tumors have also been associated with other injuries, such as those made by insects.

Nevertheless, it is not certain that wounds are essential to tumor development. In sweet clover, tumors frequently start in the pericycle lying between a legume nodule and the main vascular system of the root and, by their growth, cause the nodule to abort. There is no obvious wound present at this site.

It is supposed that some as yet unidentified wound hormone is important in mediating the effects of wounds. A synthetic growth-regulating substance, alpha-naphthalene acetic acid (NAA), when applied at 2 per cent concentration in lanolin to the epidermis, initiates a high proportion of tumors in the tips of virus-infected sweet-clover stems. Such stems contain many microscopic tumor initials composed of a limited number of cells. Apparently, most of these never develop into macroscopic tumors, but, within 72 hours after the application of NAA, proliferation of these microscopic tumors may be detected microscopically, and such growth ordinarily continues until a macroscopic overgrowth becomes obvious. Tumors stimulated to growth by the application of NAA are always associated with abortive adventitious roots. No synthetic growth hormone is known to stimulate the development of the microscopic tumors without the development of these abortive adventitious roots. Because such roots do not appear in association with normally occurring tumors, it seems certain that any hypothetical wound hormone playing a role in naturally occurring tumors must have a different composition from any of the synthetic growth regulators that have been tested. The application of the same materials to healthy stems stimulates adventitious root development but not tumors.

The wound-tumor virus is not transmissible by rubbing juice from diseased plants over the leaves of healthy plants. It is poorly transmissible when pin pricks are made through extracts from tumors rich in virus into the crowns of crimson clover plants. Only about 5 plants out of 100 are infected by the latter procedure.

In nature the virus is transmitted specifically by a few species of closely related leafhoppers, the most important being *Agallia constricta* Van Duzee and *Agalliopsis novella* (Say). Certain other closely related leafhoppers, such as *Aceratagallia sanguinolenta* (Provancher), are unable to transmit the virus. Virus may be detected in extracts by injecting small volumes (approximately 0.1 μl.) into the leafhoppers, which weigh about 1–2 mg. The virus must undergo an incubation period in the leafhopper before the insect is able to infect plants. Once the insect begins to infect plants, it may do so repeatedly over a period of several weeks. By the use of the injection technique, it was demonstrated that the tumors contain about one hundred times the concentration of virus present in the non-tumorous portion of the plant. The technique was also used to show that the virus can be diluted and transmitted from insect to insect repeatedly in series without loss of virus concentration in the insects, even though they are maintained on plants immune to the virus.

At optimum temperatures, that is, between 25° and 32° C., the minimum incuba-

tion period in the insect after the acquisition of the virus by feeding is about 2 weeks. The incubation period in the plant is of approximately the same duration. From 1 to 10 per cent of the progeny from infective females receive virus through the egg and are subsequently able to infect plants, even though they have had no chance to acquire virus from plants themselves. No symptoms of infection in the leafhoppers are known.

By means of zonal density-gradient centrifugation and electrophoresis, highly purified virus has been obtained from the plant tumors and also from the insect vector. In both cases, the virus proved to be a polyhedron about 75 mμ across.

Antisera of high titer (1/1,600) may be obtained by injection of the highly purified virus, suspended in a mineral-oil emulsion, into the muscles of rabbits. Antisera have been obtained by injection of virus purified from the plant tumors and, in another instance, purified from the insect vector. The antisera contain precipitating complement-fixing and neutralizing antibodies—the latter demonstrable in vector-injection experiments. The antisera do not react with plant protein or with insect protein in precipitin tests. Some tumor viruses of animals are claimed to incorporate host antigen. In the case of wound tumor the evidence indicates that this is not so.

In the infected plant and in the viruliferous insect, a soluble antigen related to the virus has been demonstrated. This subvirus is non-infective and does not occur in virus-free plants or insects.

Virus maintained in sweet clover for many years without access to the insect vector lost its ability to be transmitted by the insect. This vectorless virus is less virulent than the wild or vectorial virus. The former incites a smaller proportion of root tumors in relation to the total plant weight than does the vectorial virus. However, because the initial tumor reaction on the roots is less virulent, the plant makes better growth, and the total weight of tumors from the individual plant containing the vectorless virus is greater than it is from a corresponding plant containing the vectorial virus. Tumors incited by the vectorless virus contain approximately one-fourth the virus and four times the subvirus concentrations of those in tumors incited by vectorial virus.

Tumors incited on the roots of *Rumex acetosa* L. by the wound-tumor virus have been isolated and grown on a synthetic medium. This tumor tissue has an unusually high phosphate requirement and liberates an alpha-amylase into the medium in which it grows. Normal tissues grow very poorly or not at all in such a medium and do not liberate detectable amounts of the enzyme.

Sweet-clover tumors incited by the virus may be inoculated with crown-gall bacteria and will respond by the development of a superimposed crown-gall tumor. One cannot be certain that this indicates that the two agents—the virus and the bacterium—act on different cell systems to give tumefaction. The additive effects of cumulative periods of exposure to the crown-gall stimulus make this interpretation uncertain.

Wound tumor resembles virus tumors in animals not only because of the virus etiology but because of the important influence of the heredity of the host on tumor expression. It is perhaps particularly noteworthy that the virus, although it multiplies in the insect, appears to be completely innocuous therein. In sweet clover the virus incites the development of many microtumors which ordinarily do

not attain a macroscopic development, which may be stimulated by the application of certain synthetic plant growth-regulating substances. It is possible that wounds act in a similar fashion through the agency of a hypothetical wound hormone. The virus apparently changes the physiology of the plant cell so that it is able to grow in a synthetic medium which does not support, or supports only poorly, the growth of normal tissues. There is also evidence that the tumor cell liberates alpha-amylase, the liberation of which cannot be detected from corresponding normal tissue. Cultured tumor tissue requires a higher phosphate concentration in the nutrient solution than does normal tissue.

An expanded account of information in this résumé is being prepared for Volume 15 of the *Handbuch der Pflanzenphysiologie* (Berlin: Springer-Verlag).

CONCLUSIONS

By A. J. RIKER, *Chairman*

The men working with plant tumors have learned much from their associates on the animal side. While, of course, higher plants and animals are obviously different, still the basic activity of their cells is similar in many respects. This similarity was delightfully expressed many years ago by a biochemist. Let us consider the plant cell as one machine and the animal cell as another machine. Let us think, for example, of the amino acids, sugars, and vitamins as wheels in these machines. Then we see that many wheels are identical and interchangeable between the two machines. Many fundamental similarities are obvious. Only yesterday, Dr. Woolley explained the chemical relationships between serotonin in animal cells and indole-3-acetic acid in plant cells. These chemicals are related indole derivatives. They have comparable critical roles in the metabolism of cells where they occur.

The speakers today have contributed to fundamental biology. While the significance has been touched on in the different papers, perhaps for clarification I should mention a few of the trails being marked in this biological frontier for further basic research. For example:

1. Alternate pathways in nutrition and latent or unused enzyme systems are being clarified.

2. The induction of tumors is being accomplished by many different chemical, physical, and biological agents, including viruses. In certain cases the time of change from a normal to a tumor cell was determined with great precision.

3. The probability is being explored that certain tumors represent sectoral chimeras resulting from mutations of single cells.

4. A rather precise relation exists between the factors which are responsible for tumor-cell formation and the alteration in the synthetic capacity of the changed cells. The metabolism of the tumor cell is being studied in various ways to clarify how it is out of balance in comparison with that of the normal cell.

5. The development of tissue culture techniques permits the application to higher plant tissues, both diseased and normal, of the bacteriological and biochemical methods so fruitful both in microbiology and in studies of higher forms.

6. The achievement of techniques for growing single cells and of producing clones from them permits analysis of the heterogeneous population in a tumor mass. Apparently, different cells have different metabolic capacities, which will clarify much about diseased growth and differentiation.

7. While techniques are available for studying metabolic pathways, they are also available for clarifying methods for interfering with them and thus for inhibiting the pathological growth. Various common metabolic substances have inhibited growth. Likewise, the activity of a virus has been inhibited.

8. These various metabolic studies have important bearing on the activity of viruses and even of microorganisms in their interactions with host tissues.

As this symposium comes to a close, no one claims that the plant tumor problem has been solved. But at least three things must be clear: First, plants offer unique and valuable experimental materials; second, much important basic work has been done on atypical and pathological growth; and, third, excellent opportunities are at hand for further critical work and for enlarging this important biological frontier.

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